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Lymphatic vessel function in atherosclerosis

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Résumé

L'athérosclérose est une maladie inflammatoire chronique caractérisée par l'accumulation de cholestérol dans la paroi artérielle et associée à une réponse immunitaire anormale dans laquelle les macrophages jouent un rôle important. Récemment, il a été démontré que les vaisseaux lymphatiques jouent un rôle primordial dans le transport inverse du cholestérol (Martel et al. JCI 2013). L'objectif global de mon stage de maîtrise a été de mieux caractériser la dysfonction lymphatique associée à l'athérosclérose, en étudiant de plus près l'origine physiologique et temporelle de ce mauvais fonctionnement. Notre approche a été d'étudier, depuis l'initiation de l'athérosclérose jusqu'à la progression d'une lésion athérosclérotique tardive, la physiologie des deux constituants principaux qui forment les vaisseaux lymphatiques : les capillaires et collecteurs lymphatiques. En utilisant comme modèle principal des souris $Ldlr^{-/-}$; $hApoB100^{+/+}$, nous avons pu démontrer que la dysfonction lymphatique est présente avant même l'apparition de l'athérosclérose, et que cette dysfonction est principalement associée avec un défaut au niveau des vaisseaux collecteurs, limitant ainsi le transport de la lymphe des tissus périphériques vers le sang. De plus, nous avons démontré pour la première fois l'expression du récepteur au LDL par les cellules endothéliales lymphatiques. Nos travaux subséquents démontrent que ce défaut de propulsion de la lymphe pourrait être attribuable à l'absence du récepteur au LDL, et que la dysfonction lymphatique observée précocement dans l'athérosclérose peut être limitée par des injections systémiques de VEGF (vascular endothelial growth factor) –C. Ces résultats suggèrent que la caractérisation fonctionnelle de la capacité de pompage des vaisseaux collecteurs serait une condition préalable à la compréhension de l'interaction entre la fonction du système lymphatique et la progression de l'athérosclérose. Ultimement, nos travaux nous ont amené à considérer de nouvelles cibles thérapeutiques potentielles dans la prévention et le traitement de l'athérosclérose.

Mots-clés : athérosclérose, vaisseaux lymphatiques, cellules endothéliales lymphatiques, cellules musculaires lisses lymphatiques, lipoprotéines, transport cellulaire, inflammation.

Abstract

Atherosclerosis is driven by the accumulation of cholesterol in the arterial wall, which triggers an inappropriate immune response in which macrophages play an important role. It has now been shown that the lymphatic vessels play an important role in reverse cholesterol transport (Martel et al. JCI 2013). The overall objective of my Master internship was to better characterize lymphatic dysfunction associated with atherosclerosis, studying closely the physiological and temporal origin of this pathological feature. Our approach was to study, from the initiation of atherosclerosis to the progression of the atherosclerotic lesion, the physiology of the two main components that form the lymphatic vessels: the lymphatic capillaries and collectors. Using a mouse model that closely resembles human atherosclerosis ($Ldlr^{-/-}$; $hApoB100^{+/+}$) we have demonstrated that lymphatic dysfunction is present before the onset of atherosclerosis, and that this dysfunction is primarily associated with a defect in the collecting vessels, thereby limiting the lymph transport from peripheral tissues to the blood. In addition, we have clearly demonstrated, for the first time to our knowledge, the presence of the LDL receptor on lymphatic endothelial cells. Our subsequent work shows that this reduction in lymph flow could be due to the absence of the LDL receptor, and that lymphatic transport can be restored by systemic injections of VEGF (vascular endothelial growth factor) –C. These results suggest that the functional characterization of the pumping capacity of the collecting vessels would be a prerequisite for the understanding of the interactions between the function of the lymphatic system and the progression of atherosclerosis. Altogether, our work unveils new potential therapeutic targets for the prevention and treatment of atherosclerosis.

Keywords: atherosclerosis, lymphatic vessels, lymphatic endothelial cells, lymphatic smooth muscle cells, lipoproteins, cellular transport, inflammation.

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ApoA-1	Apolipoprotein A-1
ApoE	Apolipoprotein E
Ca^{2+}	Calcium ion
CaM	Calmodulin
CETP	Cholesterylester transfer protein
eNOS	Endothelial nitric oxide synthase
Erk1/2	Extracellular-signal-regulated kinase 1/2
FITC	Fluorescein isothiocyanate
hApoB100	Human apolipoprotein B100
HDL	High density lipoprotein
IDL	Intermediate-density lipoprotein
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LEC	Lymphatic endothelial cell
LV	Lymphovenous
LYVE-1	Lymphatic vessel hyaluronan receptor 1
MLC_{20}	Myosin light chain of 20 kDa
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MP	Microparticle
mRCT	Macrophage reverse cholesterol transport
MV	Microvesicle
NO	Nitric oxide
oxLDL	Oxidized LDL
P70S6K	P70S6 kinase
PCSK9	Proprotein convertase subtilisin/kexin type 9
$Pcsk9^{-/-}$	Mouse strain deficient in PCSK9
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B (also known as Akt)

PLCγ1	Phospholipase C gamma 1
PROX-1	Prospero homeobox 1
RCT	Reverse cholesterol transport
SMC	Smooth muscle cell
TPC	Total plasma cholesterol
VEGF-C	Vascular endothelial growth factor-C
VEGFR-3	Vascular endothelial growth factor receptor-3
VLDL	Very low-density lipoprotein
WT	Wild-type mouse

This mémoire is dedicated to my parents.

For their endless love, support and encouragement

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'La vie est belle' and I cannot wait for the journey ahead!

1. INTRODUCTION

Cardiovascular diseases (CVDs) are the number one cause of death worldwide. CVDs are characterized by disorders of the heart and blood vessels, and so, most deaths are from heart attacks caused by sudden blood clots in the heart's arteries[1]. Atherosclerosis is the principal cause of coronary artery disease (CAD), affecting large- and medium-sized arteries. Although atherosclerosis is a chronic inflammatory disease that remains asymptomatic for many years, its development is progressive and cumulative[2]. An advanced plaque can rupture depending on its vulnerability, often leading to the formation of a circulating thrombus that will slow down, or completely stops blood flow through heart arteries causing death of the surrounding tissues[3]. All these events can produce devastating results, such as stroke, or even worse, death.

Macrophages and cholesterol are the two main constituents driving the inflammatory response that characterizes atherosclerosis. Lipoproteins such as low-density lipoprotein (LDL) and high-density lipoprotein (HDL) play an important role in atherosclerotic lesion modulation. LDL particles get trapped in the artery wall of the vessel where they are more prone to free radical oxidation than while circulating in the bloodstream[4]. Oxidized LDL (OxLDL) further aggravate plaque formation through recruitment of pro-inflammatory factors[5]. On the other hand, HDL is a key element in atherosclerosis modulation because of its role in reverse cholesterol transport, a process that helps with cholesterol removal from plaque, and its delivery to the liver and/or the intestines for eventual excretion[6]. Therefore, to halt atherosclerosis progression, emphasis has been put on increasing the levels of HDL directly, or by inhibiting cholesterylester transfer protein (CETP), which normally transfers cholesterol from HDL to very low-density lipoprotein (VLDL) or LDL[6]. Unfortunately, the clinical outcomes revealed disappointing negative results[7-9], which made the scientific community re-think the way they approach the development of therapies that aim to promote the movement of cholesterol out of the artery wall. The identification and validation of the pathogenic mechanisms underlying this disease became a prerequisite.

New findings offer novel insight into the path that cholesterol trapped in peripheral tissues follows during cholesterol mobilization for eventual excretion outside of the body. The

lymphatic system was identified as a novel prerequisite player in the removal of cholesterol from the atherosclerotic lesion (Martel *et al*. JCI 2013)[10]. It has been shown that without a functional lymphatic network, cholesterol gets trapped in the artery wall and potentially aggravates the disease. It is suggested that the lymphatic system cleans up arteries by promoting cholesterol transport out of atherosclerotic lesions[10]. The lymphatic vessels are composed of two main components, namely the absorptive lymphatic capillaries, responsible for the uptake of cells, molecules and fluid, and the collecting vessels, characterized by pumping units (lymphangions) that are propelling the lymphatic content toward the blood circulation in a unidirectional manner[11]. The relative roles of the lymphatic capillaries and collectors in the onset of the atherosclerotic disease are still unclear.

This present work will help better delineate the specific functional roles of the lymphatic system throughout the atherosclerotic process.

1.1 Atherosclerosis

1.1.1 Pathogenesis of atherosclerosis: a multifactorial process

Atherosclerosis is the principal cause of mortality worldwide and is at the origin of most cardiovascular diseases[12]. It is a chronic inflammatory disease that affects large- and medium-sized arteries, and is now considered a multifactorial disease that involves the interplay between genetic, intrinsic and environmental factors. Atherosclerosis occurs when the arteries become clogged with fatty deposits, also called plaque, and they lose their elasticity while decreasing the lumen space, leading to reduced blood flow, or even worse, a total blockade of the vessel.

The artery wall is comprised of three different layers, which from the inside outwards, are: the intima, the media and the adventitia (Fig. 1). The intima is the thinnest and innermost layer, and it is at this level that atherosclerosis develops. It is composed of a single layer of endothelial cells displaying various properties including metabolic activities, thromboresistance, immune functions and elasticity[13]. The media is the thickest of the three layers. It is the main constituent of the artery and consists of smooth muscle cells (SMCs) surrounded by an extracellular matrix composed of elastic and fibrous proteins (collagen and elastin). In general, the media is avascular, except its outer regions, which are perfused by the *vasa vasorum* of the adventitia (tunica externa)[13]. The latter is the external layer of the blood vessel wall that consists of loosely organized connective tissue rich in collagen and elastic fibers, as well as fibroblasts and adipocytes. The adventitia ensures the anchorage of the arteries to the surrounding structures and is sometimes also traversed by longitudinal smooth muscle fibers[13].

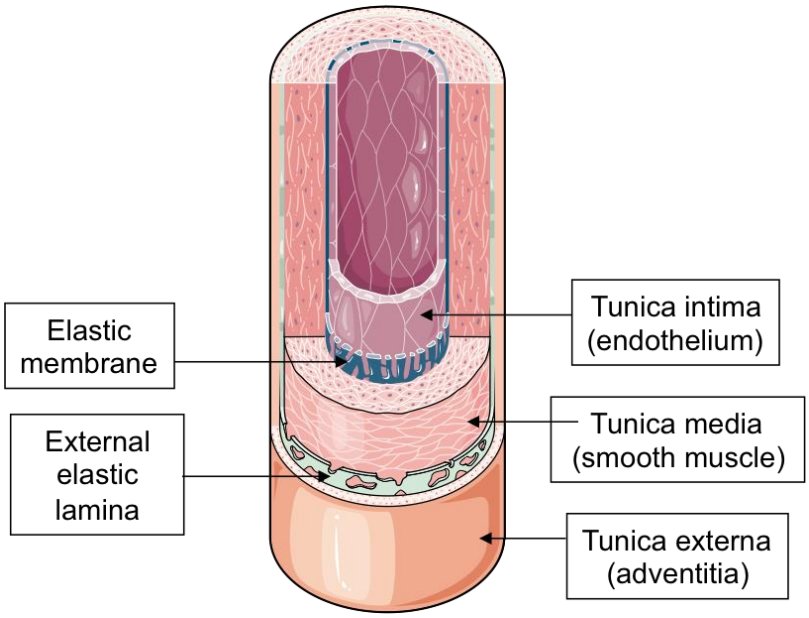

Figure 1. Structure of the artery wall.

Illustration showing the different layers of a blood vessel wall. Adapted from Servier Medical Art http://www.servier.com/slidekit/?item=16

Atherosclerotic plaque formation is a continuous process and can extend around the entire circumference of the vessel. Plaque development is slow, gradual and remains asymptomatic for a long time[5]. Monocyte-derived macrophages and resident macrophages accumulate in the aortic intima[14]; they engulf lipids and become foam cells, and produce a wide-ranging spectrum of inflammatory mediators[15-17]. The physiological mechanism through which cholesterol homeostasis is obtained in the artery wall, characterized by its mobilization from foam cells and subsequent transfer from the peripheral tissues to the liver and the intestines for further excretion, is called macrophage reverse cholesterol transport (mRCT)[18]. The removal of cholesterol from foam cells and plaques is essential for the reduction of atherosclerosis burden and plaque rupture[19].

1.1.2 Lipid metabolism and its role in atherosclerosis

Triglycerides, cholesterol and different lipoproteins are well known to be involved in the pathogenesis of atherosclerosis[20]. A high ratio of HDL: LDL in the body is associated with

a lower risk of CAD[21], and both entities play a pivotal, yet opposite role, in the modulation of atherosclerosis.

LDL is directly involved in the development of atherosclerosis, mostly due to its accumulation in the endothelium of the blood vessel. The required receptor for the endocytosis of cholesterol-rich LDL is found mainly at the surface of hepatocytes and is called the LDL receptor (LDLR). If not enough of these receptors are present, cholesterol uptake by the cells is reduced, leading to increased cholesterol in the blood vessels[22]. A commonly known genetic disorder, familial hypercholesterolemia, is characterized by the inability of LDL particles to be removed from the body and therefore leads to a severe increase in the risk of cardiovascular disease[23]. LDL is composed of apolipoproteins, phospholipids, triglycerides and cholesterol. Its most important and atherogenic component is apolipoprotein B (ApoB), more precisely apoB100, which is synthetized by the liver. It is this outer phospholipid layer of the LDL particles that binds to LDLR[24]. ApoB100 is also present in other lipoproteins such as VLDL, intermediate-density lipoproteins (IDL) and chylomicrons. However, the main protein of IDL and chylomicrons is apolipoprotein E (ApoE), which is primarily produced by the liver and macrophages, and also mediates cholesterol metabolism[25]. The initial retention of LDL in the vessel wall, followed by its oxidation, is the primary event in the early stages of an atherosclerotic lesion development, and this oxLDL promotes further recruitment and retention of monocytes that become macrophages. The protein portion of LDL will then be modified, which will lead to its impaired recognition by the LDLR and instead, increase its recognition by scavenger receptors and oxLDL receptor that are not regulated by cholesterol content in the cell[26,27]. Overall, this leads to overaccumulation of cholesterol, and both of these derivatives will go on to activate endothelial cells, SMCs and macrophages, which in the end will facilitate vasoconstriction, thrombosis and platelet aggregation. Furthermore, the uptake of oxLDL by these monocyte-derived macrophages will lead to the formation of foam cells in the subendothelial space, and in the end, all these support the formation of the atherosclerotic lesion[28].

Interestingly, LDLR is tightly regulated by a proprotein convertase subtilisin/kexin type-9 (PCSK9), which binds to it and targets it for lysosomal degradation in cells, leading to decreased hepatic clearance of plasma LDL-cholesterol (LDL-C). In addition to modulating

cholesterol transport and metabolism, PCSK9 also promotes intestinal overproduction of triglyceride-rich apoB lipoproteins[29]. However, since PCSK9 interferes with the clearance of LDL-C from the blood, its loss-of-function mutations are associated with up to 85% lower plasma LDL-C levels[30] and offer significant protection from coronary heart disease, such as atherosclerosis[31]. PCSK9 is now considered a great target for cholesterol-lowering therapies[32]. In the largest monotherapy trial using a PCSK9 inhibitor to date, Evolocumab reduced LDL-C from baseline by 55% to 57% more than placebo and 38% to 40% more than ezetimibe, which lowers cholesterol absorption from the intestine and was well tolerated in patients with hypercholesterolemia[33]. Furthermore, as statin intolerance has been a major limitation in the use of statins, a first and only study of a new class of LDL-C-lowering agents in patients selected with a rigorously documented intolerance to statins showed promising results. Alirocumab reduces LDL-C levels by approximately 50% when used as monotherapy, and has so far shown a safety profile comparable with ezetimibe or placebo[34]. On July 24, 2015 the first PCSK9 inhibitor, Alirocumab was approved by the FDA as a second line of treatment for adults with high cholesterol not controlled by diet and statin treatment. On August 2015, the FDA approved a second PCSK9 inhibitor, Evolocumab, for patients who are unable to get their LDL cholesterol under control with current treatment options. Moreover, PCSK9 has also been shown to affect the regulation of epithelial Na^+ channel (ENaC) trafficking and therefore modulate epithelial Na^+ absorption that is critical for blood pressure control[35]. Therefore, PCSK9 inhibition could be effective by its direct modulation of LDL-C uptake by the liver, but it could also potentially be associated with pleiotropic effects. So far, PCSK9 inhibition has an excellent safety profile and promises to provide a well-tolerated and effective therapeutic measure against coronary heart disease (CHD)[36].

Another key element in atherosclerosis is HDL, which is the smallest of the lipoprotein particles, but it is the densest because it contains the highest proportion of proteins to lipids. Its most abundant apolipoprotein is apolipoprotein A-1 (ApoA-1)[37]. Most protective effects of HDL are mediated by its cell surface receptors. HDL counteracts the proatherogenic activity of LDL by mobilizing cholesterol from the arterial intima and delivering it to the scavenger receptor class B1 (SR-B1), present on the surface of the liver, for further elimination into the bile[38]. In humans, one of the most relevant pathways is the indirect one, which is mediated by

CETP, a protein that exchanges triglycerides of VLDL for cholesteryl esters of HDL and leads to the processing of VLDLs to LDL, which will then be removed from circulation by the LDLR pathway[39]. Furthermore, it has been shown that HDL can function as an acceptor, transporter and inactivator of oxLDL, and is also responsible for the inhibition of monocyte adhesion in the intima of the blood vessel, preserves endothelium-dependent vascular activity through its effect on endothelial nitric oxide synthase (eNOS) and prevents thrombosis[40]. Therefore, HDL particles are important because unlike the larger particles, such as LDL, they transfer cholesterol away from cells, artery walls and tissues, through the bloodstream, back to both LDL particles, as well as back to the liver for excretion.

The inverse relationship between HDL-cholesterol levels and CHD incited a lot of interest in pharmacological agents that elevate plasma levels of HDL. Statins are first-line drugs for the treatment of dyslipidemias and CAD prevention due to their ability to lower plasma LDL-C levels[41]. This treatment focuses mainly on endogenous LDL-C, and results in neglect of other important aspects of lipoprotein metabolism[42]. Significant side effects such as severe muscular pain are also displayed in most patients[43]. As statins offer little to no effect on increasing HDL levels, the attempt was then focused on other ways to increase these levels, such as directly through the inhibition of CETP, which normally transfers cholesterol from HDL, to VLDL or LDL[44]. Examples include HDL-raising drugs such as torcetrapib, which ended up showing an increase in cardiovascular risk[45], as well as dalcetrapib, that simply lacked effectiveness[46]. Niacin, or Vitamin B3, are other effective HDL-raising agents currently on the market that first emerged as dyslipidemia treatment[47]. As a result, new ways to approach treatments, particularly those aimed at preventing plaque development in atherosclerosis, are currently under close investigation, and as we will soon see in the sections to come, there are promising venues ahead.

1.2 The lymphatic system

1.2.1 General anatomy and functions of the lymphatic system

The lymphatic system is part of the circulatory system and plays a vital role in host defense and adaptive immunity. Although the roles of lymphatic vessels in tissue maintenance and disease are now well known, their origins are still a subject of debate. As far back as 1902, Florence Sabin, based upon results obtained by India ink injection experiments in pigs, proposed that isolated primitive lymph sacs, which are precursors of the lymph vessels, originated from endothelial cells that bud from the cardinal vein during early development[48]. Interestingly, Klotz *et al*. found that the lymphatic endothelial cells (LECs) that form lymphatic vessels in the heart originate both from embryonic veins and from other non-venous sources, like the yolk sac[49]. Therefore, contrary to previous belief, it has been proven that a significant part of the dermal lymphatic vasculature forms independently of sprouting from veins[50]. Many different transcription factors regulate lymphatic development, one of the most crucial being Prospero-related homeobox-1 (Prox1)[51]. Cells that are $Prox1^+$ depend on a paracrine factor called VEGF-C to be able to spread away from the embryonic veins. The vascular endothelial growth factor receptor-3 (VEGFR-3) serves as a receptor for lymphatic-specific VEGFs, VEGF-C and VEGF-D. VEGF-C is important for normal development of the lymphatic vessels and Prox1 drives VEGFR-3 expression that enables LECs to respond to VEGFR-3 ligands[52]. In zebra fish, it has been shown that VEGF-D can compensate for the absence of VEGF-C, making the latter dispensible[53]. Separation of lymphatic fluid from blood requires platelet aggregation[54]. Platelets regulate the blood/lymphatic vessel separation by inhibiting the proliferation, migration, and tube formation of LECs, upon activation by C-type lectin-like receptor 2 (CLEC-2)/podoplanin interaction. Interaction of podoplanin present on LECs with CLEC-2 present on platelets triggers a signalling cascade leading to platelet aggregation and the formation of fibrin-containing platelet thrombi that protect both the lymphovenous junction (LV) and the thoracic duct from backward flow[55].

Characterized by a network of vessels that carry a clear fluid called lymph, the lymphatic system is now recognized as working in close collaboration with the cardiovascular

system, but unlike the circulatory system, it is not a closed system[56]. One of its main roles is to maintain fluid homeostasis in the body with the help of its thin-walled and blind-ended lymphatic capillaries[57]. These initial lymphatics help absorb the ultrafiltrates from peripheral tissues, they are highly permeable and are constituted of specialized, discontinuous 'button-like' junctions between endothelial cells[58]. Lymphatic capillaries are characterized by the absence of SMCs and at the surface of the LECs they specifically express lymphatic vessel endothelial hyaluronan receptor (LYVE-1). Following its absorption by the lymphatic capillaries, lymph will move on to converge into larger pre-collecting vessels, to subsequently reach the collecting lymphatic vessels[57,59]. Lymphatic capillaries have a sparse and discontinuous basement membrane and they lack pericytes, which are the contractile cells that wrap around the endothelial cells of blood capillaries and venules throughout the body. Anchoring filaments attach LECs to the extracellular matrix and prevent vessel collapse under conditions of increased interstitial pressure. Both "buttons" and "zippers" are composed of adherens and tight junction–associated proteins like VE-cadherin, zonula occludens-1, occludin, and claudin-5 (Fig. 2). The main difference between them resides in their organization[60]. Collecting lymphatic vessels are covered with continuous basement membrane and SMCs, and are characterized by the expression of podoplanin. Therefore, collecting vessels are contractile lymphatics that propel lymph in a unidirectional manner, with the help of intraluminal bi-leaflet valves, as well as smooth muscle walls. The functional unit of a collecting lymphatic vessel is called a lymphangion, representing the segment between two valves. All the lymph collected from the entire left side of the body, the digestive tract and the right side of the lower part of the body flows into a single major vessel, the thoracic duct. The latter empties into the left subclavian vein. The lymph in the right side of the head, neck, and chest is collected by the right lymph duct and empties into the right subclavian vein. The two subclavian veins will then drain into the blood circulation through the LV, right near their junction with the internal jugular veins[59].

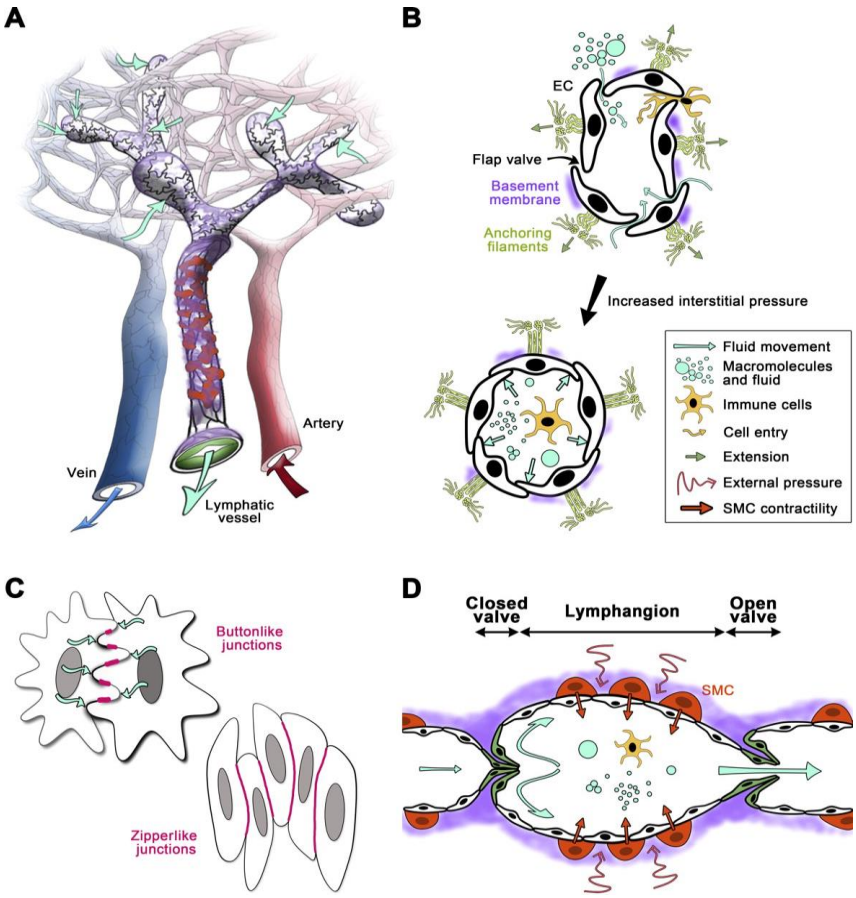

Figure 2. Organization of the lymphatic vasculature.

(A) The lymphatic vasculature is responsible for the absorption of fluid, macromolecules, and cells from the interstitium. (B) Mechanism of lymph formation in capillaries. Interstitial components penetrate lymphatic capillaries via openings between LECs. The specialized structure of such openings prevents the return of lymph back to the interstitium. Anchoring filaments attach LECs to the ECM and prevent vessel collapse under conditions of increased interstitial pressure (black arrow). (C) Junctional organization of LECs in lymphatic capillaries and collecting vessels. Both "buttons" and "zippers" share a repertoire of adherens and tight junction–associated proteins, like VE-cadherin, zonula occludens, occludin, and claudin-5. The main difference between them resides in their organization[58]. (D) Mechanism of lymph propulsion in collecting vessels. Coordinated opening and closure of lymphatic valves is important for efficient lymph transport. SMCs covering each lymphangion possess intrinsic contractile activity. Schulte-Merker S, *et al*. Lymphatic vascular morphogenesis in development, physiology, and disease. J Cell Biol 2011; 193(4): 607-18.

1.2.2 The physiology of contraction

Largely aqueous with relatively low concentrations of proteins and cells compared to blood, lymph is propelled through the collecting lymphatics via two primary mechanisms: 1) intrinsic, characterized by active pumping as a result of lymphatic muscle cell contraction, the lymphangions and the valves; 2) extrinsic, compression mechanisms such as the movement of skeletal muscle or other tissues surrounding the lymphatics, as well as respiration. Coordinated opening and closure of lymphatic valves is important for efficient lymph transport. As previously discussed, lymphangions are wrapped in a disorderly manner by lymphatic smooth muscle cells[61]. Unlike vascular smooth muscle, lymphatic muscle is characterized by both a rapid, phasic contractile activity that drives the intrinsic lymphatic pumping, as well as a slower, tonic form of contractions seen in blood SMCs. The uniqueness of lymphatic contractility is due to the lymphatic muscle being composed of both smooth and striated muscle[62]. However, lymphatic valve function, which operates in an open–close manner, is passive and responds to the pressure difference between pre- and post-valve lymphagions[63]. In contrast, lymphangion contraction is an active process that requires the generation of force by SMCs, which are mainly dependant on myocyte intracellular Ca^{2+} levels. In this regard, a subset of muscle cells acts as pacemaker cells, initiating and driving propagation of the Ca^{2+} wave to other cells downstream, inducing a series of action potential-like spikes of calcium that cause the synchronized contractions of lymphangions[64]. Contraction by the lymphangions is an alternate process, and at the same time, it is normal for two or more adjacent lymphangions to contract and relax at the same time (Fig. 3)[65].

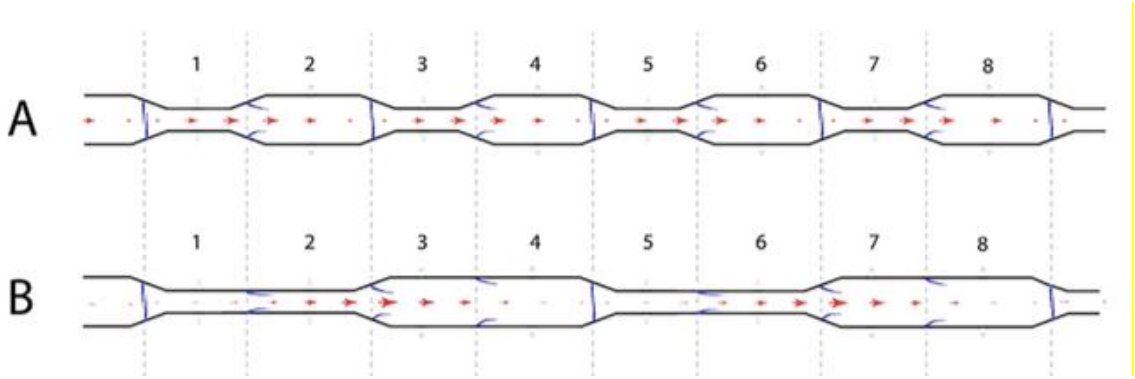

Figure 3. Examples of lymphangion coordination.

(A) Contractions might alternate in adjacent lymphangions. (B) It is possible that two or more adjacent lymphangions contract and relax together. Adapted from: Munn LL. Mechanobiology of lymphatic contractions. *Semin Cell Dev Biol* 2015; **38**: 67-74.

Several mechanisms trigger and modulate vessel phasic or tonic contraction by influencing lympatic SMCs cytoplasmic Ca^{2+} levels. Modulation of the 20-kDa myosin light chain (MLC_{20}) is an important factor of lymphatic contractile strength. MLC_{20} phosphorylation is regulated by the activities of myosin light-chain kinase (MLCK) and myosin light-chain kinase phosphatase (MLCP). Lymphatic muscle contraction is initiated by an increase in cytosolic Ca^{2+} levels resulting in Ca^{2+} binding to the universal intracellular Ca^{2+} receptor protein, calmodulin (CaM), which will activate the catalytic subunit of MLCK. MLCK then phosphorylates MLC_{20}, leading to lymphatic vessel contraction. MLC_{20} phosphorylation is reversed by MLCP, resulting in lymphatic muscle relaxation[66]. The best-known and most studied mechanism for interfering with SMC contraction is nitric oxide (NO), which acts at multiple points of the Ca^{2+} contraction pathway. NO produced by eNOS regulates systemic blood pressure, vascular remodelling and angiogenesis[67]. Shear stress is the most important physiological stimulus of its production and phosphorylation of eNOS by Akt represents a Ca^{2+}-independent regulatory mechanism for activation of eNOS. When it comes to the lymphatic system, once produced, NO can diffuse to the SMCs enveloping the lymphatic vessels and affect pumping. It works by modulating Ca^{2+} release and uptake[68], as well as the enzymes responsible for force production[69,70]. NO decreases vascular tone through a cGMP-dependent mechanism, which leads to reduced intracellular Ca^{2+}, by inhibiting its

release from internal stores. Conversely, NO can activate several other channels to increase Ca^{2+} efflux[71]. NO also induces relaxation through its direct inhibitory effect on the MLC[72]. Overall, NO is a vasodilator that opposes the Ca^{2+} response of the lymphatic vessels, leading to dilatation and decreased contraction frequency[73]. Hence, the lymphatic system is under the constant influence of various factors that modulate its contractility, controlling its crucial roles both as conduit and pump.

1.2.3 Lymph composition in lymphatic function

Lymph composition is changed during its flow from the periphery, with its protein concentration increasing along the lymphatic vessels[74]. Since lymph is derived from the interstitial fluid, its composition constantly changes depending on its interaction between the surrounding cells and blood. Blood and the interstitial fluid are in dynamic equilibrium with each other, meaning that water and solutes can pass between the two by diffusion across gaps in capillary walls called intercellular clefts. Although peripheral lymph lipoproteins have been characterized in animals, information about their composition is limited, and their ultrastructure is nearly unexplored. Studies analyzing human lymph have started to emerge, and they have confirmed that lymph composition is different than that of plasma or serum[10]. Back in 2000, Nanjee *et al*. demonstrated that the concentration of small pre-beta HDLs in human tissue fluids is determined only in part by their transfer across capillary endothelium from plasma. They showed that the local production of pre-beta HDL in the periphery, by remodelling of spheroidal HDLs in tissue fluids, is just as important[29]. Continuing down this path, in 2001, Nanjee *et al*. examined the composition, as well as the ultrastructures of different subclasses of normal human peripheral lymph lipoproteins. Total cholesterol concentration in lymph HDL was 30% greater in lymph than could be explained by the transendothelial transfer of HDL from plasma, which provided direct confirmation that HDL acquire cholesterol in the extravascular compartment[30].

Extracellular vesicles (EVs) are plasma membrane-derived vesicles released from cells upon activation or during apoptosis. Cellular EVs in body fluids constitute a heterogeneous population, differing in cellular origin, numbers, size, antigenic composition and functional properties[75]. Despite being considered simple cellular debris for the longest time, it is now well documented that EVs can interact with neighboring cells and are suspected to be key

players in many physiopathological processes such as thrombosis, autoimmune diseases and inflammation[76]. Due to their diversity, EVs are considered important biomarkers and thus, their precise detection in several biological fluids, such as lymph, is important to better understand all their different functional activities[77]. As they are an important vector of information exchange between cells of different origins, in the course of their various interactions they cause structural and functional changes, especially at the level of the vascular wall, in the endothelium[78]. Although EVs are present in the peripheral blood of healthy individuals, marked elevations occur in many disease states[76], as they have been shown to be a major component of atherosclerotic plaques, especially platelet-derived EVs[75]. Therefore, in a pathological setting, particularly atherosclerosis, it is important to understand why and how the presence of EVs is affected, by studying lymph composition.

We now know that a broad array of cytokines, proteins, growth factors, lipoproteins and maybe even extracellular vesicles are contained within lymphatic fluid, which play an important role in metabolism, proliferation, as well as immunoregulation[76]. We have yet to see just how much effect they may have on the lymphatic vessels themselves.

1.3 Lymphatic vessels in inflammation and cardiovascular diseases

1.3.1 Mouse models of lymphatic dysfunction

Several models with impaired lymphatic function have started to emerge. Primary congenital lymphedema (Milroy disease) is a rare autosomal dominant condition caused by mutations in the *vegfr-3* gene[79]. Primary human lymphedema is characterized by a chronic and disfiguring swelling of the extremities. A popular mouse model to study the physiological regulation of lymph flow and to assess the therapeutic potential of VEGF-C to stimulate lymphatic revascularization has been put forth by Alitalo *et al*[80]. Chy mice have an inactivating VEGFR-3 mutation in their germ line and swelling of the limbs because of incomplete development of lymphatic vessels within the dermis. Furthermore, promising therapeutic results were observed when they used virus-mediated VEGF-C gene therapy, as they were able to generate functional lymphatic vessels in lymphedema mice[81]. Another example is the $Prox1^{+/-}$ mouse, which accumulates fat as a consequence of lymphatic vascular leakage, and has been used as a new model for adult-onset obesity and lymphatic vascular disease. Functional inactivation of a single allele of the Prox1 gene led to adult-onset obesity due to abnormal lymph leakage from mispatterned and ruptured lymphatic vessels[82]. Another interesting mouse model includes the microsurgical ablation of the lymphatic vessels in the tail of the mouse, which results in lymph stagnation, lymph vessel dilation (with a marked increase in tail volume), accumulation of fibroblasts, fat, and skin cells, impaired clearance of immune cells from the tail, and profound accumulation of inflammatory cells[83]. It is a useful model that closely imitates some key features of acquired lymphedema in humans, and in combination with a diverse array of mouse genetics, it could lead the way to the better understanding of the molecular basis of lymphedema. All these different mouse models have not only helped us to improve our understanding of different lymphatic-related pathologic conditions and their relationship with inflammation, but also to re-evaluate the functional roles of the lymphatic vascular network.

1.3.2 Lymphatic function in atherosclerosis

Despite the well-defined roles of the lymphatic system in preserving fluid balance throughout the body by returning plasma proteins from interstitial spaces back to the blood circulation, its function in heart disease has lately been getting much attention. Alterations in the intestinal lymphatic network are well-established features of human and experimental inflammatory bowel disease (IBD)[84]. As lymphatic vessels play an essential role in intestinal lipid uptake, impairment of lymphatic vessel function leads to enhanced adipose tissue accumulation in patients with lymphedema and in genetic mouse models of lymphatic dysfunction[85]. Interestingly, Blum *et al*. showed that adipose tissue expansion due to a high-fat diet leads to functional impairment of the lymphatic vasculature, mainly at the level of the collecting vessels. In the heart, evidence shows that blocking cardiac lymph flow may contribute to several forms of cardiac injury including cardiac lymphedema and poor heart performance in animal and human heart studies[86]. It was back in 1981 that Lemole observed intimal vessel thickness following lymphatic vessel blockage. He suggested that the accumulation of interstitial fluid in the artery wall could be due to a phenomenon called lymphostasis, which may contribute to the development of atherosclerosis due to factors present in the intimal edema[87]. Thirty years later, fundamental studies and clinical studies started to emerge in this regard. Studies analyzing the morphology of lymphatic vessels in the artery wall allowed for insights between their associations with atherosclerosis. In animal models, lymphatic vessels have been observed in the adventitia of the artery wall[88]. In fact, Xu *et al*. associated the presence of lymphatic vessels within the adventitia of the artery wall an important factor for the draining of local inflammatory cells and cytokines from peripheral tissues[89]. In a clinical setting, Drozdz *et al*. took interest in the presence of lymphatics in the adventitia of the internal carotid artery in humans and showed that the number of adventitial lymphatics increases with the severity of atherosclerosis measured as intimal thickness[90]. Furthermore, they showed that arteries with a dense network of lymphatic vessels seem to be naturally protected against atherosclerosis when compared to those without such a network[91]. Martel *et al*. introduced a new integrated model of mRCT in which they clearly showed that without a functional lymphatic network, cholesterol cannot be properly conducted out of the artery wall. They used a surgical model of aortic transplant from a hypercholesterolemic ApoE

deficient ($ApoE^{-/-}$) donor to a hypercholesterolemic $ApoE^{-/-}$ receiver in which ApoE vector was injected to induce cholesterol efflux, and showed that the pattern of the newly regrown lymphatic vessels post-transplant is influenced by the blood flow through the transplant[10]. The lymphatic vessels thus formed appeared to be atheroprotective: in conditions where lymphatic vessels had fully grown post-transplant, the cholesterol contained in the transplanted artery was able to exit the atherosclerotic lesion. By contrast, partial inhibition of lymphatic regrowth using VEGFR-3 antibody was reflected by retention of cholesterol in the artery wall of the transplanted aortic segment[10]. Along the same path, Vuorio *et al*. published that lymphatic impairment worsened the atherosclerotic plaque formation in atherogenic $Ldlr^{-/-}/ApoB^{100/100}$ mice crossed with transgenic mice bearing localized lymphatic insufficiency (sVEGFR-3 or Chy), and analyzed the effects of the absence of lymphatics on lipoprotein metabolism and atherosclerosis[92]. The group observed a positive correlation between atheroma formation and the absence of lymphatic vessels, contrary to previous studies that showed opposite effects. This study further questions whether lymphatic vessels are beneficial or detrimental. Furthermore, these $Ldlr^{-/-}/ApoB^{100/100}$ mice crossed with transgenic mice bearing localized lymphatic insufficiency (sVEGFR-3 or Chy) have increased cholesterol levels leading to accelerated atherogenesis, which suggests that lymphatic vessels play a crucial role in the maintenance of proper lipoprotein metabolism and overall vascular homeostasis[92].

Based on the newly described integrated model for mRCT[10], it is believed that within the atherosclerotic lesion, cholesterol ester (CE) is exported from macrophages and uploaded onto cholesterol acceptors like HDL, which will then cross the medial layer of the artery to reach the adventitia. From there, it will enter the adventitial lymphatic capillaries and get propelled through the afferent collecting lymphatic to the draining lymph node. It passes through the efferent collecting lymphatic to enter the bloodstream at the level of the subclavian vein[10]. The next steps are well studied and imply an uptake of the circulating cholesterol by the liver, via SR-B1 receptor[93], where most cholesterol is transformed into bile acids and secreted into the bile for excretion. In parallel, CE from HDL can also be transferred in VLDL and LDL, *via* CETP[94], a pathway that is not present in mice, and internalized by the liver *via* LDLR[94,95], to also be secreted into the bile. Hence, it is crucial to redirect more

therapies towards improving lymphatic function, which should facilitate cholesterol clearance and limit inflammation, all in the hopes of preventing or reversing atherosclerosis.

2. RESULTS

2.1 General mémoire objective

The general objective of my M.Sc. project was to better delineate the functional roles of the lymphatic system before atherosclerosis onset and its progression. More specifically, I have studied the effect of *in vivo* modulation of the LDLR on lymphatic function.

Based on published data by Dr. Martel[10], we were able to pursue her work with newly formed hypotheses of our own. This new data I have helped acquire is now the stepping-stone to many upcoming projects currently being developed in our laboratory. Our first manuscript is presented in section 4.2.

2.2 Presentation of the article

In this article, we built on previously published results by Dr. Martel[10], as well as the literature, to study the origin of the lymphatic dysfunction seen in atherosclerosis. The objective was to see at which level of the lymphatic vessel functional impairment originates and at what point during atherosclerosis development the defect becomes prominent.

We characterized thoroughly in different mouse models the morphology and functionality of the lymphatic capillaries, and it gave us no reason to doubt that lymphatic dysfunction must surely originate at a higher level, namely the collecting vessels. Our next step was to better grasp the cellular composition of these vessels, and that is how we came to show that the LDLR is present even at the surface of LECs. As LDLR, whose expression is down-regulated by physiologic levels of PCSK9, is a major player in atherosclerosis, we aimed to investigate whether and how PCSK9 and LDLR modulation plays a role in lymphatic function.

We subsequently observed that in a mouse model with an over-accumulation of LDLR, lymphatic function increased throughout age, when compared to wild type. In contrast, even before development of plaque, in an $Ldlr^{-/-}$; $hApoB100^{+/+}$ mouse model (also called ATX), an acute lymphatic dysfunction occurred. This new finding opened doors to the possibility of new

therapeutic targets such at VEGF-C, which we demonstrate may exert a rescue effect when treating young $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice, before atherosclerosis onset.

Overall, our results suggest that:

- Lymphatic transport tends to improve with age in $Pcsk9^{-/-}$ mice compared to WT
- LDLR is expressed on LECs and associated with lymphatic vessel function
- Collecting lymphatic vessels may be responsible for the impairment in lymphatic function in $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice
- VEGF-C systemic treatment abrogates the lymphatic dysfunction that is observed before atherosclerosis onset in $Ldlr^{-/-}$; $hApoB100^{+/+}$

Participation of each author of the article:

AM: Project conceptualisation, troubleshooting, methods validation, experiments, data analysis and manuscript writing

FD: Experiments and data analysis

GM: Resources (Pcsk9$^{-/-}$ mice), scientific consultation

CM: Project conceptualisation, troubleshooting, methods validation, experiments, data analysis and manuscript writing

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Effects of LDL Receptor Modulation on Lymphatic Function

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ABSTRACT

Atherosclerosis is driven by the accumulation of immune cells and cholesterol in the arterial wall. Although recent studies have shown that lymphatic vessels play an important role in macrophage reverse cholesterol transport, the mechanisms regulating this pathological feature remain unknown. In the current report, we aim to better characterize this lymphatic dysfunction associated with atherosclerosis by studying the physiological and temporal origins of this impairment. We observed that mice deficient in Pcsk9 had improved lymphatic function throughout age when compared to WT mice for up to six months, while displaying enhanced expression of LDLR on lymphatic endothelial cells. Then, using a mouse model ($Ldlr^{-/-}$; $hApoB100^{+/+}$) that closely resembles human atherosclerosis we show that lymphatic dysfunction is present before atherosclerosis lesion formation, and that this dysfunction is primarily associated with a defect in the lymphatic collecting vessels. Systemic treatment with a selective VEGFR-3 agonist (VEGF-C 152s) rescued this impairment in a cholesterol-independent manner. Taken together, our results suggest for the first time that the absence of PCSK9 is associated with improved lymphatic function and unveil new potential therapeutic targets for the prevention and treatment of atherosclerosis.

Introduction

Low-density lipoprotein receptors (LDLR) are important players in atherosclerotic lesion development and progression mostly through lipoprotein metabolism regulation[1], as demonstrated in patients with familial hypercholesterolemia[2]. This genetic disorder is most often caused by reduced function of LDLR, apolipoprotein B (apoB) or gain-of-function mutations in protein convertase subtilisin kexin type 9 (PCSK9), resulting in a severe elevation of the plasma levels of LDL[3]. Circulating LDL-cholesterol (LDL-C) accumulates in the artery wall of blood vessels and leads to premature atherosclerosis[4]. ApoB100 is the apolipoprotein and ligand of LDLR found in lipoproteins synthesized by the liver, and is the sole protein of LDL[5]. LDLR is present on the outer surface of many types of cells[6]. Hepatocytes are important cells bearing LDLR, as the liver is responsible for removing most excess cholesterol from the body, through LDL uptake[5]. PCSK9 is a well-established down-regulator of LDLR[7], which acts by binding to the receptor causing its lysosomal degradation in cells. Since PCSK9 interferes with the clearance of LDL-C from the blood, its loss-of-function mutations are associated with up to 85% lower plasma LDL-C levels[8] and offer significant protection from coronary artery disease (CAD), like atherosclerosis[9]. On the contrary, high-density lipoprotein (HDL) reduces cardiovascular risk, mainly due to its role in macrophage reverse cholesterol transport (mRCT), promoting cholesterol removal from plaque and its eventual excretion by the liver and/or the intestines[10,11]. In order to halt atherosclerosis progression and decrease the prevalence of CAD, emphasis has logically been put on improving this physiological process. However, most of the different ways used to increase HDL levels did not demonstrate any clinical benefits, and did not lead to improved mRCT or decreased CAD[12-14]. Consequently, such conclusions made the scientific community re-think the way they approach the development of therapies aimed to increase mRCT and favourably modulate atherosclerosis.

PCSK9 inhibition has an excellent safety profile in clinical trials and promises to provide a well-tolerated and effective therapeutic measure against CHD[15]. PCSK9 inhibition could act via its direct modulation of LDL-C uptake by the liver, but it could also potentially be associated with pleiotropic effects. Recent findings show that PCSK9 deficiency increases

CD36 levels in the liver and adipose tissue[16] and reduces liver metastasis by its ability to lower cholesterol levels[17]. Moreover, PCSK9 has been shown to affect the regulation of epithelial Na^+ channel (ENaC) trafficking and therefore modulate epithelial Na^+ absorption, which is critical for blood pressure control[18]. Lastly, PCSK9 was shown to be expressed endogenously both at the mRNA and protein level in murine peritoneal macrophages[19]. Giunzioni *et al*. showed for the first time that this PCSK9 expression directly influences atherosclerotic plaque composition with no changes in serum cholesterol levels, which suggests a direct effect of macrophage PCSK9 in inflammation and plaque development[20].

An additional new therapeutic target in atherosclerosis has recently arisen. In a recent study, the lymphatic system has been identified as a novel prerequisite player in the removal of cholesterol from the atherosclerotic lesion by mRCT[21]. It has been reported that without a functional lymphatic network, cholesterol cannot leave the artery wall and might potentially aggravate the disease. Accordingly, it is now suggested that cholesterol leaves tissues and reaches the bloodstream by first entering lymphatic vessels (LVs), putting forward a new integrated model for mRCT[21,22]. The blood vasculature and the lymphatic systems are parallel and interdependent networks[23]. In contrast to the blood vascular network, the lymphatic vascular network is an open, unidirectional and low-pressure vascular system. Lymphatic development and regulation are dependent mostly upon VEGF-C/D and its receptor VEGFR-3[24]. The LVs are composed of two different entities, bearing distinct but complementary roles. Lymph is first absorbed through thin-walled and blind-ended initial lymphatics (also called lymphatic capillaries), which are highly permeable and are constituted of specialized, discontinuous "button-like" junctions between endothelial cells[25]. Expression of lymphatic vessel hyaluronan receptor 1 (LYVE-1) on the LEC and absence of smooth muscle cells (SMC), are characteristics of the lymphatic capillaries[25]. Following, lymph moves from the lymphatic capillaries into collecting vessels, the entities responsible of maintaining lymph flow through contraction of units called lymphangions. Collecting vessels resemble small veins, but have bi-leaflet valves between the contractile units in order to prevent back flow. They are also characterized by a basement membrane, down-regulation of LYVE-1 expression, podoplanin expression, continuous "zipper-like" cell-cell junctions and a discontinuous SMC layer[26]. Lymph is propelled away from the periphery most of the time

against a hydrostatic pressure gradient primarily *via* the phasic and synchronized contractions of the lymphangions, mediated by the intrinsic contractility of SMC, the contraction of surrounding skeletal muscles, and arterial pulsations[27-31].

Since lymphatic function is now linked to atherosclerosis, and the LDLR plays a central role in cardiovascular disease and exhibits modulation following PCSK9 inhibition treatment, studying the implication of PCSK9 on lymphatic function has become of great interest. Herein, we sought to better characterize the interplay between lymphatic function and the onset and progression of atherosclerosis by exploring the possible link between lymphatic function and LDLR modulation in $Pcsk9^{-/-}$ and $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice. We investigate whether and how the absence of PCSK9 and, subsequently, increased LDLR protein expression has a beneficial effect on lymphatic function, and further portrays the premises of the chronological sequence of atherosclerosis-associated lymphatic dysfunction.

Results

Lymphatic vessel function is enhanced in $Pcsk9^{-/-}$ mice.

As PCSK9 targets and mechanisms of action are now known not to be solely confined to the liver, we sought to investigate its effect on lymphatic function. We herein hypothesized that knocking out Pcsk9 may positively modulate lymphatic transport. To address this, we first assessed the capacity of popliteal lymphatic vessels to carry Evans blue (EB) dye from the initial lymphatics located in the dermis of the foot pad up to the collecting lymphatics and the corresponding draining popliteal lymph node (LN) (Fig. 1a, upper panel; Supplementary Fig. S1 online). Intradermal injection of EB dye revealed that the dye intensity was greater within the dominant[32] collecting vessel of $Pcsk9^{-/-}$ mice, with no or little interruption on its path (Fig. 1b, lower panel, green arrow *vs*. red arrows). This observation suggests that lymphatic collecting vessel function was improved in $Pcsk9^{-/-}$ mice when compared to atherosclerosis-prone $Ldlr^{-/-}$; $hApoB100^{+/+}$ and even WT mice. In addition, less extravasated EB dye was detected in the surrounding adipose tissue of $Pcsk9^{-/-}$ mice when compared to that of $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice, signifying an improved lymphatic collector function.

As a complementary measure of lymphatic function, we evaluated the efficiency of lymphatic vessels to transport dendritic cells from the peripheral tissue to the corresponding draining LNs, using the well-described FITC painting assay[33]. Similar to the improved EB dye transport, the migration of skin dendritic cells ($CD45^+CD11c^+FITC^+$) in $Pcsk9^{-/-}$ mice (Fig. 1b) was improved when compared to WT mice at 6 months. Analysis of the back skin from that same region (Supplementary Fig. S2a online) revealed that the adipose tissue layer tends to be thinner in 3-month-old $Pcsk9^{-/-}$ mice when compared to WT mice (Supplementary Fig. S2b online), but not in 6- (Supplementary Fig. S2c online) and 12- (Supplementary Fig. S2d online) month-old $Pcsk9^{-/-}$ mice.

Initial lymphatic vessel morphology and number are unchanged in Pcsk9$^{-/-}$.

As hypercholesterolemia-associated lymphatic dysfunction in 16-week-old $apoE^{-/-}$ mice is associated with initial lymphatic hyperplasia[34], we conversely sought to investigate whether the absence of PCSK9 would be associated with morphological changes within initial lymphatics. We first assessed initial lymphatic vessel ($Lyve\text{-}1^+$) morphology and density (Fig. 2a) by looking at their diameter (Fig. 2b), their number (Fig. 2c) and the total surface area occupied by the vessels (Fig. 2d) in the mouse ear dermis. No significant changes were observed in the athero-protected mouse model when compared to WT mice in an age-dependent manner. In order to confirm our finding in the artery wall, we investigated the presence of initial lymphatic in the adventitia of the aortic sinus (Fig. 2e), a blood vessel layer where lymphatic vessels have been consistently observed[35].Once again, no difference was seen at any age when looking at the morphology or density of $Lyve\text{-}1^+$ vessels (Fig. 2f-h).

LDLR is expressed on lymphatic endothelial cells of popliteal collecting vessel.

Based on our findings that lymphatic function is improved in $Pcsk9^{-/-}$ mice without apparent modulation in initial lymphatic morphology or growth, we sought to investigate whether collecting LVs could thus be responsible for the observed related lymphatic gain-of-function observed in this $Pcsk9^{-/-}$ mouse model. To begin with, we hypothesized that LDLR *per se* could act as an important modulator in lymphatic collecting vessel function, as it would be drastically increased on LVs compared to WT mice. Our initial step was to detect the

presence of LDLR on collecting LVs and then test its modulation by PCSK9. Therefore, for the first time to our knowledge, we have shown that LDLR was expressed on popliteal lymphatic collecting vessels (Fig. 3a). Immunofluorescence was performed and demonstrated the presence of LDLR on podoplanin^{+} LECs, but to a lesser extent smooth muscle cells (SMC, Fig. 3a). Immunofluorescence (Fig. 3b) and western blots performed with equal amount of protein loading (Fig. 3c) revealed that Pcsk9$^{-/-}$ mice displayed a LDLR overaccumulation phenotype when compared to WT and Ldlr$^{-/-}$ mice.

Lymphatic dysfunction appeared before atherosclerosis onset and decreased during its progression in Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice.

As our data are bridging lymphatic function to LDLR modulation on lymphatic collecting vessel ECs through PCSK9, we next conversely investigated whether decreased LDLR levels *per se* could be a premise to lymphatic collecting vessel dysfunction. Atherosclerosis-prone Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice are severely dyslipidemic, exhibit premature blood endothelial dysfunction, oxidative stress and inflammation at 3-month-old[36-38].They spontaneously develop aortic atherosclerotic lesions after 4 months while on regular chow diet. Before that age, they do not have atherosclerotic lesions, but as they get older, they become increasingly atherosclerotic (Supplementary Fig. S3a, S3c, S3d online). Resembling the human atherosclerotic phenotype due in part by the addition of the human apoB100 transgene, these mice are a suitable model to study in further detail factors that could lead to atherosclerosis or at least impact its progression. Lymphatic transport assays revealed a significant (p<0.001) lymphatic function impairment in 3-month-old Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice (Fig. 4a) that are not yet bearing atherosclerotic lesions (Supplementary Fig. S3a, S3c, S3d) or macrophages in the artery wall (Supplementary Fig. S3b and S3e online). As these mice are highly dyslipidemic, we wanted to test whether this severely increased plasma cholesterol content *per se* would be reflected by an enhanced lymphatic transport defect. To test this, we have measured lymphatic function in both young (3-month-old) preatherosclerotic Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice and Ldlr$^{-/-}$ mice. As total cholesterol content of Ldlr$^{-/-}$ mice is known to be half the concentration retrieved in the Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice (~200 mg/ml vs. ~700 mg/ml)[37,39,40], one could expect to see an even more impaired lymphatic function in the latter group. However, as a premise of the effect of LDLR *per se* on lymphatic function as a proatherosclerotic factor, we

observed no significant difference between the two groups (Fig. 4a). Furthermore, lymphatic dysfunction became even more apparent ($p<0.05$, at 12 mo) throughout atherosclerosis progression (Fig. 4b). Just like in apoE$^{-/-}$ mice in which dermal lymphatic vessel diameter increases in conjunction with dyslipidemia[34], aging Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice displayed enlarged initial lymphatic vessels (Fig. 4e, $p<0.0001$, between 6- to 12-month), increased total Lyve-1^{+} area (Fig. 4f, $p<0.001$, at 12-month) and decreased Lyve-1^{+} vessels number. As mentioned previously, in contrast to Pcsk9$^{-/-}$ mice (Fig. 1a), Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice demonstrated interrupted EB dye presence (red arrows), as well as extravasated dye from these vessels (yellow arrows). This further supported our showcased lymphatic impairment following FITC painting, and was also a first indication of collecting vessel malfunction. As Pcsk9$^{-/-}$ mice abundant in LDLR display improved lymphatic transport and as 3 month-old pre-atherosclerotic Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice lacking LDLR already showed a lymphatic dysfunction that was exacerbated in parallel to lesion formation, these results reinforce the idea that the LDLR *per se* could play a direct role on lymphatic function.

VEGF-C 152s treatment rescues lymphatic function in young preatherosclerotic Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice

VEGF-C was proven to reverse hypercholesterolemia-associated lymphatic dysfunction in apoE$^{-/-}$ mice, and to stimulate lymphatic pumping *ex vivo* in a model of rat mesenteric lymphatics by a VEGF receptor-3-dependent mechanism[27]. Therefore, as our results pointed out that LDLR mediated-lymphatic function modulation, at least through PCSK9 variation, would most likely be due to an effect on lymphatic collecting vessels, we then investigated whether and how lymphatic dysfunction can be restored in young Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice. VEGF-C 152s is a point mutant that only binds to and activates signaling through VEGFR-3, and unlike wild type VEGF-C, is unable to bind VEGFR-2. VEGF-C treatment has previously been shown to restore lymphatic function in mice with established atherosclerosis[41], and we herein aimed to assess whether treatment with VEGF-C 152s could rescue lymphatic function before the onset of atherosclerosis in 3-month-old pre-atherosclerotic Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice. Following treatment, a significant ($p<0.001$) increase in lymphatic cellular transport was observed (Fig. 5c), despite no difference seen in dextran-Cy5 absorption by the initial lymphatics (Fig. 5d). Furthermore, when assessing the number of Lyve-1^{+} vessels in the

adventitia of the aortic sinus (Fig. 5a), a significant (p<0.05) increase when compared to control (PBS-treated) mice was observed (Fig. 6b). Lastly, in order to exclude the fact that VEGF-C could have decreased total plasma cholesterol (TPC) and subsequently improved lymphatic function, TPC was measured in VEGF-C 152s treated $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice versus controls (PBS treated $Ldlr^{-/-}$; $hApoB100^{+/+}$). Unexpectedly, our results showed that TPC in our PBS-treated $Ldlr^{-/-}$; $hApoB100^{+/+}$ was even significantly higher in VEGF-C-treated mice versus PBS-treated mice (Fig. 5e).

Discussion

Based on original data from the Framingham Study indicating that low plasma levels of HDL-C are associated with premature CAD[42], increasing levels of HDL has become an approach of great interest in research aiming at better controlling related pathologies such as atherosclerosis. However, recent resulting conclusions have met with disappointing clinical outcomes, emphasizing that atherosclerosis is a multifactorial disease and that many different avenues need to be considered in order to better control the atherosclerotic process and to prevent CAD. In the past decade, tremendous progress has been made to better characterize the interplay between the lymphatic network and chronic inflammatory diseases[41], including atherosclerosis[43]. Studies analyzing the morphology of lymphatic vessels in the artery wall allowed for insights into their associations with atherosclerosis. In animal models, lymphatic vessels have been observed in the adventitia of the artery wall[44]. In fact, Xu *et al*. demonstrated the presence of lymphatic vessels within the adventitia of the artery wall to be an important factor for the draining of local inflammatory cells and cytokines from peripheral tissues[45]. In a clinical setting, Drozdz *et al*. took interest in the presence of lymphatics in the adventitia of the internal carotid artery in humans and showed that the number of adventitial lymphatic vessels increases with the severity of atherosclerosis measured as intimal thickness[46]. Along the same path, Vuorio *et al*. recently published that lymphatic impairment worsened the atherosclerosis plaque formation in atherogenic $LDLR^{-/-}/ApoB^{100/100}$ mice crossed with transgenic mice having lymphatic localized insufficiency, and analyzed the effects of the absence of lymphatics on lipoprotein metabolism and atherosclerosis[47]. This

group observed a positive correlation between increased atheroma formation and the absence of lymphatic vessels. It is now well established that lymphatic vessels are more abundant in the adventitial layer of the arterial walls in both animals and humans[48].

With this perspective, we here combine recent knowledge governing the identification of two attractive new therapeutic targets in atherosclerosis, and sought to characterize their possible interaction with the ultimate goal of limiting the disastrous consequences related to this pathology. PCSK9 has emerging therapeutic roles in dyslipidemia-associated diseases, particularly atherosclerosis and new evidence shows that LVs play a key role in controlling cholesterol efflux from peripheral tissues, such as the atherosclerotic lesion[21]. In this study, we aimed to better delineate the effects of the modulation of LDLR, particularly through PCSK9 inflection, on lymphatic function. Our results suggest for the first time that the absence of PCSK9 is associated with improved lymphatic function, most likely targeting the collector segment of the vessel. Lymphatic collecting vessel function is disturbed at a very early stage before the onset of atherosclerosis and linked to LDLR absence, and this defective transport can be prevented by enhancing VEGF-C/VEGFR-3 specific binding. Although the related specific mechanisms remain to be understood, our data suggest that LDLR *per se* could have a functional role in the early stage of lymphatic dysfunction, thus corroborating a pleiotropic effect of PCSK9 inhibition in atheroprotection.

On top of its positive effect on plasma cholesterol uptake by LDLR, our results suggest that lymphatic function also greatly benefits from downregulation of PCSK9. The limited subcutaneous adipose tissue accumulation we observed in $Pcsk9^{-/-}$ mice opposes the increased visceral adipogenesis previously reported that is most likely due to VLDLR[49] and/or CD36[16], and their role in TG regulation. Whereas the regulation of the latter could be considered in our findings, we cannot exclude a potential role of the lymphatic vessels in the clearance process of the adipose tissue in peripheral sites, such as the skin. Using two independent techniques, our first set of results demonstrate that lymphatic function is enhanced in $Pcsk9^{-/-}$ mice, when compared to WT mice. This effect peaks on the 6^{th} month of age, and seems to diminish by the 12^{th} month, suggesting that PCSK9 inhibition could abrogate the aging-related lymphatic dysfunction up to a certain stage. It was recently documented by Dixon's group that a

preferential drainage pattern in lymphatic vessels is possible through what they call "dominant vessels", and in a smaller proportion in "non-dominant vessels"[32]. They bring forward the interesting hypothesis that dominant vessels are driven by extrinsic factors, while non-dominant vessels are under the influence of intrinsic contraction. Our EB technique further supports this affirmation, as illustrated (Fig. 1a). PCSK9 may act upon LDLR present on lymphatic collecting vessels, leading to a cellular signaling pathway that modulates the capacity of these collectors to properly contract. Along these same lines, we believe PCSK9 may both have a similar effect and also disturb the anti-atherogenic properties of LECs, such as the modulation of eNOs and prostacyclin production, as well as levels of oxidative stress. As NO produced by the endothelium acts as a vasoactive agent that modulates Ca^{2+} release and uptake to control lymphatic contraction frequency[50], we are aiming to study in further details the Ca^{2+} fluxes and NO production patterns in our atherosclerosis-prone and protected mice. As aging is a potential player in lymphatic dysfunction[51], the tendency of losing their protective effect by 12 months of age could thus be attributable to this factor. $Pcsk9^{-/-}$ mice exhibit normal initial lymphatics, with no visible changes seen in the morphology and number, at any age. Since at the initial lymphatic level no changes were observed, despite the improvement in dendritic cell transport and EB dye, we believe modifications due to LDLR enhancement may originate in the collecting vessels. Subsequently, it was necessary to demonstrate the ubiquitous nature of LDLR by assessing its presence on endothelial lymphatic collecting vessel cells. Despite a reduced lymph flow, Le May et al. observed that PCSK9 expression impacts postprandial triglyceridemia[52], another important cardiovascular risk factor. As they observed an increased presence of LDLR in the intestines, it should be taken into consideration that there might be an increase of LDLR at the surface of the lacteal vessels (intestine lymphatic vessels) thus playing a role in lymphatic transport *per se*.

Although plaque development becomes apparent only by the age of 4 to 6 months[53] in the $Ldlr^{-/-}$; $hApoB100^{+/+}$ mouse model, we observed lymphatic dysfunction as early as in 3-month old mice, before any sign of lesion formation. In parallel, lymphatic dysfunction and atherosclerosis continues to worsen in concert with age. Contrarily, when assessing capillary functionality in 3-month old pre-atherosclerotic mice by a molecular absorption technique (Cy5-Dextran), no differences in molecular lymphatic transport were noted. For that reason,

similar to the Pcsk9$^{-/-}$ mouse model, we went on to investigate the morphology and number of the initial lymphatics present in the ear dermis, and observed a decrease in their number, which we assume to be age-related[51]. However, their diameter is significantly increased by the age of 12 months. This could be due to lymphatic capillary hyperplasia, not consequent of a change in their morphology, but rather, due to a dysfunction at the collecting vessels level. As these initial vessels are larger, they occupy more space in the dermis, despite their decrease in number. Our results are further supported by systemic lymphatic hyperplasia of the initial lymphatics in hypercholesterolemic apoE$^{-/-}$ mice[34]. As observed in atherosclerosis, lymphedema is characterized by inflammation, fibrosis, and adipose deposition[54]. All these adverse events, together with the added effects of aging in lymphatic collectors[51], cause impairment in lymph transport, fluid homeostasis and pathogen clearance[55]. One thing is definite; LDLR and PCSK9 may play a crucial role in better understanding this impairment, and emphasizing the need for further studies. Both models offer great insight into potential atherosclerosis treatments. While PCSK9 biology has demonstrated a significant new way of offering protection against CAD, Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice present a humanized atherosclerosis-prone model that aids the study of the progression of atherosclerosis, and even its potential prevention, as seen with VEGF-C 152s.

The potential predisposition to development of atherosclerosis, seen in Ldlr$^{-/-}$; hApoB100$^{+/+}$ mice suggests the use of VEGF-C 152s, which selectively binds solely to VEGFR-3, as a therapeutic alternative. VEGF-C has been shown to reduce lymphedema by stimulating lymphangiogenesis[29,30,56]. Indeed, we report here that the defect observed in cell migration through the lymphatics is significantly improved following treatment with the mutant form of VEGF-C, VEGF-C 152s, in young pre-atherosclerotic mice. This could be explained by the fact that physiologically, VEGF-C may either enhance lymphangiogenesis or stimulate lymphatic pumping, which is a more recent concept[27]. Several *in vitro* studies have revealed that VEGF-C/VEGFR-3 binding on LEC induces activation of PI3K/Akt and results in phosphorylation of eNOS[30]. Like VEGF, VEGF-C has vasoactive- and endothelial barrier-altering properties[57-59], and it has recently been shown that its binding to VEGFR-3 can alter the intrinsic and phasic pumping of collecting LVs[27]. VEGF-C stimulates lymphatic pumping *ex vivo* in a model of rat mesenteric lymphatics[27]. Therefore, we believe that the impairment of

lymphatic transport, which occurs even before the onset of atherosclerosis, is potentially attributable to the contractile capacity of the collecting LVs. Since it has been well established that hypercholesterolemia impairs lymphatic function[34], it was important to demonstrate that the impaired lymphatic function observed in $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice was not only due to hypercholesterolemia, but also due to a lack of LDLR *per se*. Three-month-old $Ldlr^{-/-}$ mice have a TPC that ranges between 200 to 370 mg/dl[37,39,40], whereas TPC of the $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice included herein, whether treated with VEGF-C 152s or not, is much higher (1170 or 860 mg/dl, respectively). Despite this major difference in cholesterol levels, both 3-month old $Ldlr^{-/-}$ and $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice show similar impairment in lymphatic transport. Furthermore, regardless of the increase in plasma cholesterol observed in VEGF-C treated $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice, lymphatic function was significantly improved compared to control (PBS treated $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice). Once again, this shows that in the context of atherosclerosis, hypercholesterolemia is not the sole player affecting lymphatic function.

Taken together, our results suggest for the first time that the absence of PCSK9 is associated with improved lymphatic function. Lymphatic collecting vessel function is disturbed at a very early stage before any sign of atherosclerotic lesion formation, and this defect can be prevented by systemic VEGF-C treatment. We believe that our report reveals new important information regarding the specific interactions between the lymphatic system and cardiovascular diseases, thus opening doors to potential therapeutic targets for the prevention and treatment of atherosclerosis.

Materials and Methods

Mice. C57BL/6 wild-type (WT) and $Pcsk9^{-/-}$ mice (backcrossed against C57BL/6 for 10 generations at our facility) were from Jackson Laboratories. A knockout/transgenic dyslipidemic $LDLR^{-/-}$; $hApoB^{+/+}$ (ATX) mouse colony was established by one of our collaborators at the Montreal Heart Institute (Dr. Éric Thorin)[36,37]. All experiments were carried out on 3-, 6- and 12- month-old male and female mice. Differences between sexes were not observed. Animals were housed in a pathogen-free environment under a 12 hour

light-dark cycle with free access to water and to standard chow diet. Mice were euthanized with CO_2 and perfused with 15 ml phosphate buffered-saline (PBS). All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Montreal Heart Institute Animal Care Committee.

Experimental setup. Lymph nodes (LNs), ears, aortas, hearts and popliteal lymphatic collecting vessels were harvested and either freshly processed for flow cytometry analysis and/or western blots, or fixed in 4% paraformaldehyde (PFA) for future analysis, as described below. In some experiments, three month-old ATX mice received an intraperitoneal cavity injection of 25 ng VEGF-C 152s (purified recombinant Rat VEGFC protein (152s), Fitzgerald) dissolved in PBS, three times a week for 4 weeks. The control group received PBS alone.

Lymphatic Functional Assessment. Lymphatic function was first assessed by quantifying the dermal clearance of dextran by the initial lymphatics, as described previously[60]. Briefly, a total of 1 µl fluorescent (Cy5) dextran (70 kDa) at a concentration of 2 mg/ml in sterile PBS was injected intradermally in the ear pinnae of anesthetized mice. Due to its large size, the tracer is specifically uptaken by blind-ended lymphatic capillaries avoiding absorption by blood capillaries. Fluorescence decay was observed through the skin using a fluorescence stereomicroscope (Leica M205) and images of the skin were acquired every minute for 30 minutes. The rate of clearance was determined by calculating the area under the curve (AUC) of fluorescence intensity at each time point, and normalized to the initial value. The normalized rate of fluorescence decay was then calculated from the slope of AUC *vs*. time, which is considered proportional to the actual rate of Cy5-dextran clearance. Second, lymphatic function was also assessed using Evans blue dye for tracing popliteal lymphatic vessels[61]. Mice were anesthetized with isoflurane and following Evans Blue intradermal injection in the footpad, popliteal lymphatic collecting vessels were visualized using a Stereo Discovery V8 microscope (Zeiss). Third, migration of dendritic cell to LN was evaluated after epicutaneous application of a FITC solution as described previously[62]. The animals were sacrificed 18 hours later and corresponding skin-draining LNs were recovered and enzymatically digested with collagenase D for 25 min at 37°C. Cells were then passed through

a 70 μm cell strainer, washed, counted, and stained for analysis by flow cytometry (BD Biosciences LSR II). Conjugated antibodies CD11b PerCp-Cy5.5, CD11c PeCy7 and CD45-APC were used (BioLegend).

Primary mouse lymphatic endothelial cell immunofluorescence and immunoblotting analysis. Popliteal lymphatic collecting vessels were identified following Evans blue intradermal injection as described above, and harvested. For analysis of LDLR expression on mouse primary lymphatic endothelial cells, popliteal lymphatic collecting vessels were digested and proteins were extracted following incubation with radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. Proteins from lymphatic collecting vessels (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by its transfer to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature. Membranes were incubated with an antibody against LDLR (Novus Biologicals) overnight at 4^{o}C, and HRP-conjugated secondary antibodies were used for detection using the Western Lightning Ultra chemiluminescence kit (PerkinElmer). Experiments were performed by pooling of 3 to 4 mice *per* experimental group. The presence of LDLR was also confirmed on isolated popliteal lymphatic collecting vessels of mice by whole-mount immunofluorescence analysis following incubation with anti-podoplanin (Angio Bio Co.) and anti-LDLR antibodies. Images were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63x/1.4 oil dic objective. Images were deconvolved using a theoretical point spread function and Huygens Professional software (Scientific Volume Imaging) and volumes were rendered using the surface renderer option.

Quantification of initial lymphatic density. Hearts and aortae were removed and fixed in 4% PFA for 2 hours. Hearts were transferred into PBS containing 30% sucrose (wt/vol) overnight at 4°C before being immersed in OCT compound and stored at -80°C. Eight-micrometer-thick cryosections of the aortic sinus were prepared. Cross-sections of the aortic sinus were stained with anti-LYVE-1 (ABCAM) and anti-CD68 (Biolegend) antibodies, and then incubated with the appropriate secondary antibodies. As macrophages can also be positive for LYVE-1, adventitial lymphatic capillaries were identified as LYVE-1^{+}CD68^{-} cells

forming vessel-like shapes. Whole-mount immunohistochemical analysis of the ear dermis to visualize lymphatic vessels was performed as described previously[34]. Ear dermis were stained for lymphatic capillaries (anti-LYVE-1, ABCAM) at 4°C, and then sections were incubated with Alexa Fluor 647 conjugated donkey anti-rabbit antibody and Cy3 donkey anti-rat (Jackson ImmunoResearch). All imaging was performed on a Fluoview FV10i microscope (Olympus). All vessel counts were performed by one observer. The relative quantification of the number of lymphatic capillaries (LYVE-1^+ vessels), their diameter and the total surface area they occupy was determined by computer-assisted morphometric analysis. Neutral lipid assessment in atherosclerotic lesions was performed by Oil-red-O (ORO) staining (Sigma).

Immunohistochemistry. The back skin of the animals was shaved and harvested, fixed in 10% formalin, and embedded in paraffin. Eight-micrometer thick back-skin sections were stained with hematoxylin and eosin (H&E). Pictures were taken with an Olympus B45 microscope and visualized using ImagePro Plus 7.0 software. All image handling was performed using ImageJ software.

Plasma cholesterol quantitation. Blood was collected into heparin-containing tubes by cardiac puncture and plasma was obtained following centrifugation at 2400g for 10 minutes. To avoid thawing-related damage on lipoprotein conformation, 5% sucrose (in PBS) was added before samples were frozen. Total plasma cholesterol was measured using a Wako kit, according to manufacturer instructions (Wako).

Statistics. Data are expressed as the mean ± SEM. Statistical differences were assessed using a two-tailed non-parametric Student's *t* test, unless otherwise stated, with $p < 0.05$ reported as statistically significant, using Prism software version 6.0c (GraphPad). All experiments contained three or more replicate mice per experimental parameter.

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Author contributions statement

AM and CM designed and carried out experiments, analyzed data, and wrote the manuscript. FD carried out experiments and GM contributed to design of experiments and revised the manuscript.

Competing financial interests

The authors declare no competing financial interests. This work was partially supported by the Montreal Heart Institute Foundation (CM, GM), the Banting Research Foundation (CM) and the Canadian Institutes of Health Research (CIHR) (MOP133598) (GM). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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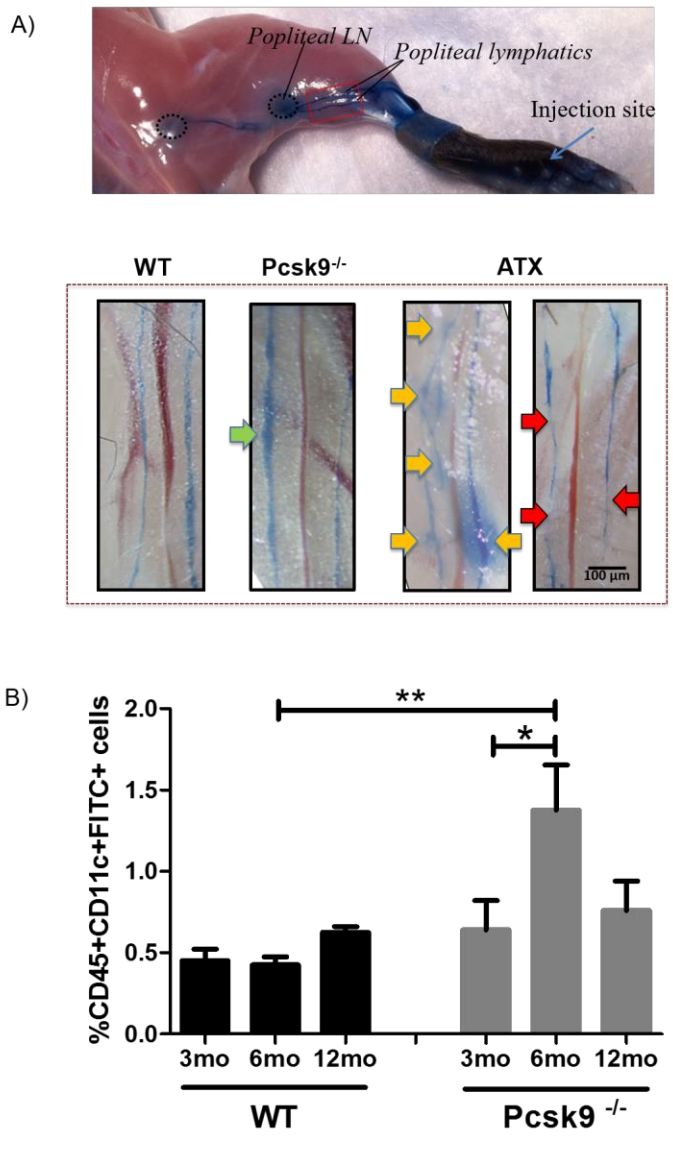


Figure 1. Lymphatic function is enhanced in Pcsk9$^{-/-}$ mice. A) Lymphatic vascular function was assessed following Evans Blue intradermal injection in the footpad of 6-month-old WT, Pcsk9$^{-/-}$ and ATX mice. After 30 minutes, lymphatic vessels were visualized using a Stereo Discovery V8 microscope. Pictures were taken using a Canon Rebel XSI camera. Pcsk9$^{-/-}$ mice show a higher intensity of Evans Blue within the vessel (green arrow), and clear surroundings. In contrast, ATX mice demonstrate interrupted Evans Blue presence (red arrows), as well as extravasated dye from these vessels (yellow arrows). Similar results were obtained in at least three repeated experiments. B) Dendritic cell migration, examined 18h after a contact sensitization assay (FITC painting) was measured by quantification of %CD45^{+}CD11c^{+}FITC^{+} cells from lymph nodes of 3-, 6- and 12- month-old WT and Pcsk9$^{-/-}$ mice. Experiments were performed using 5-14 replicates per experimental group (mean ± SEM). Analysis was performed using a BD LSRII flow cytometer. *p<0.05 and **p<0.01.

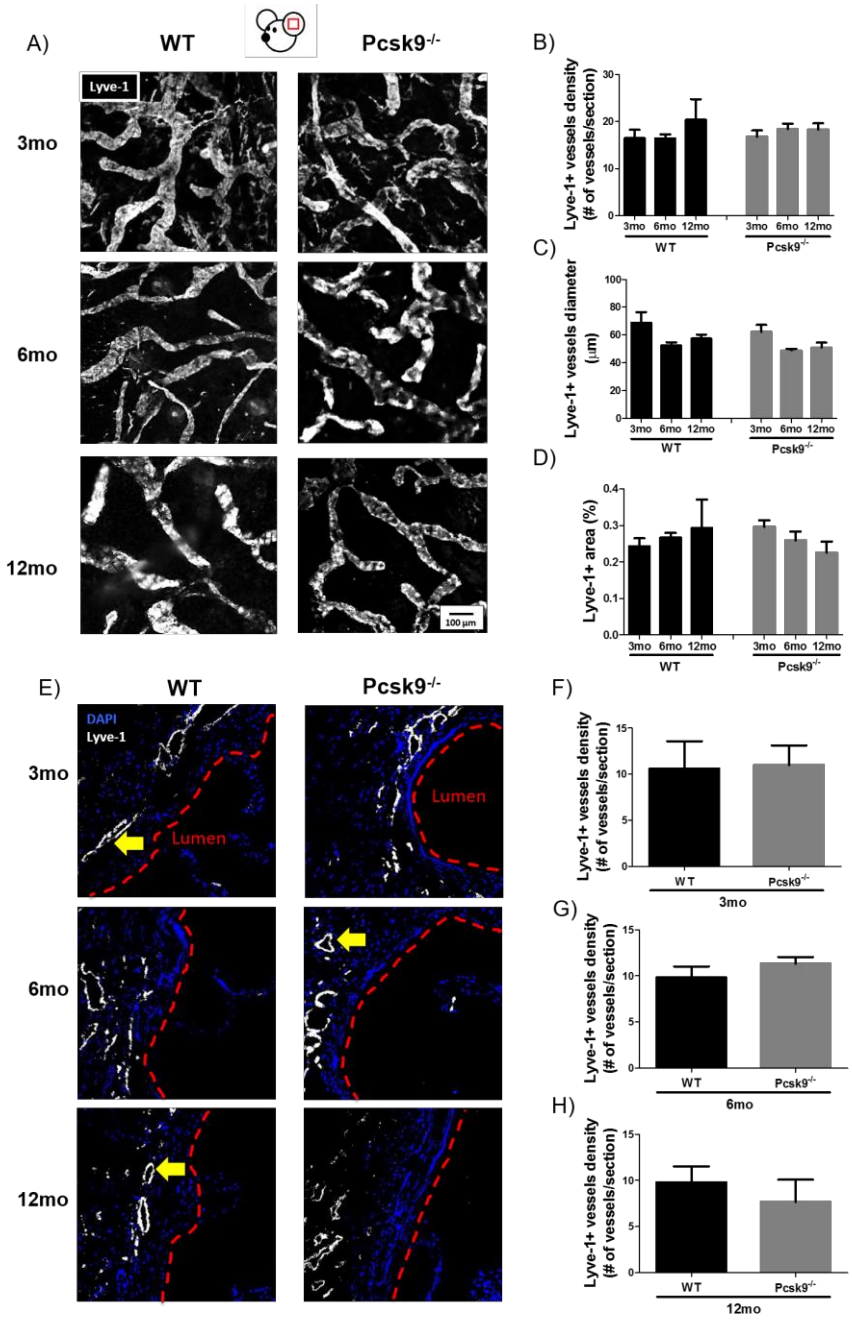
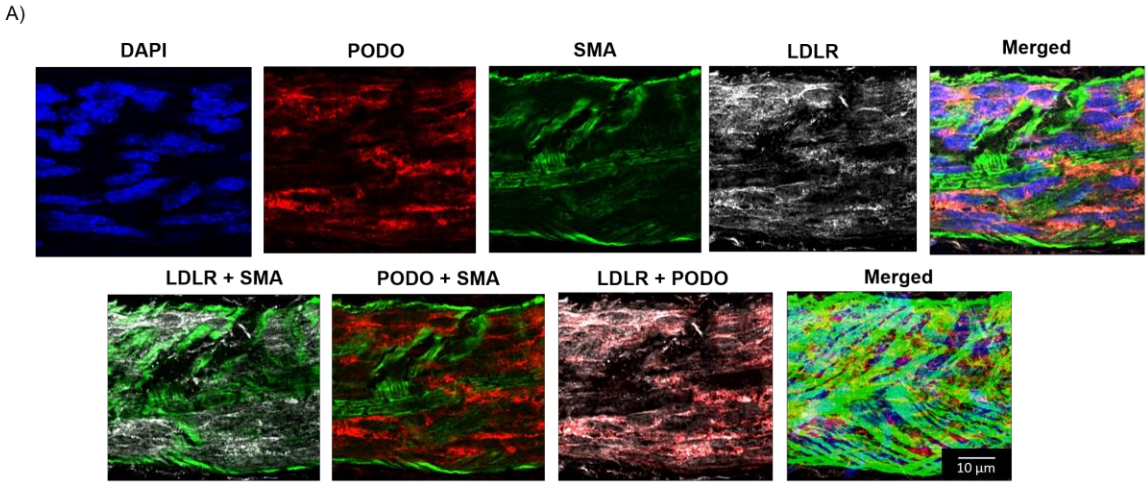
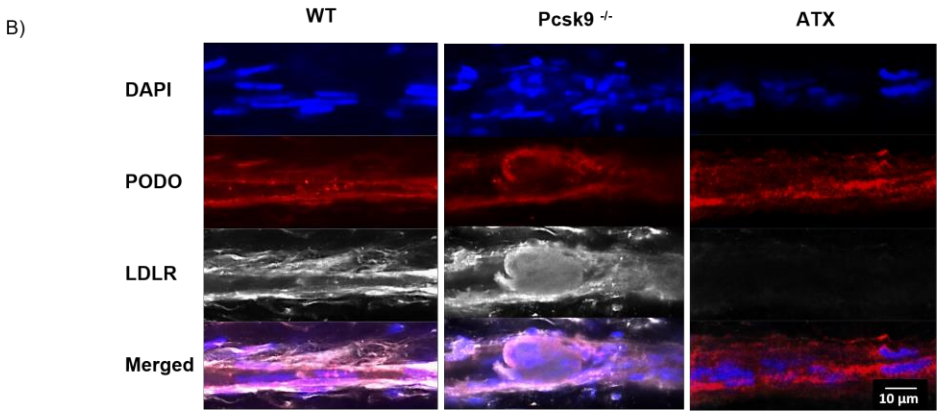
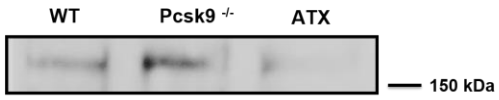


Figure 2. Initial lymphatic vessel number, diameter, and area is unchanged in Pcsk9$^{-/-}$ mice. A) Immunofluorescence of initial lymphatic vessels (Lyve-1^{+}) in the ear dermis of 3-, 6- and 12-month-old WT and Pcsk9$^{-/-}$ animals. Quantification of the B) number C) diameter and D) surface area of Lyve-1^{+} vessels in the ear dermis. Experiments were performed using 5-9 replicates per experimental group (mean of 5 measurements/slide ± SEM). E) Immunofluorescence of initial lymphatic vessels (Lyve-1^{+}) and DAPI in the aortic sinus adventitia in 3-, 6- and 12-month old WT and Pcsk9$^{-/-}$ mice. Only Lyve-1^{+} vessels of any continuous tube-like shapes formed of Lyve-1^{+}CD68^{-} were included, as indicated by the yellow arrows. Quantification of the number of Lyve-1^{+} vessels/aortic section at F) 3-, G) 6- and H) 12-month mice. Experiments were performed with 3-6 replicates per experimental group (mean of 2-3 measurements/slide ± SEM). Pictures were taken using Fluoview FV10i confocal microscope (Olympus). All image handling was performed using ImageJ software.





C)



	WT	*Pcsk9-/-*	ATX
Protein lysate concentration (mg/ml)	1.10	1.22	1.14
	1.11	1.54	0.87
	1.40	1.36	1.32
Volume (µl)	27.24	24.51	26.31
	27.11	19.51	34.41
	21.42	22.04	22.79

Figure 3. LDLR is expressed on lymphatic endothelial cells of popliteal collecting vessel and its level is modulated by PCSK9. A) LDLR expression on smooth muscle cells (smooth muscle actin, SMA) and LEC was detected by immunofluorescence of DAPI, podoplanin (PODO) and LDLR in longitudinally imaged single plan in popliteal lymphatic collecting vessels of WT mice. In the lower right panel, z-stacks were acquired and deconvolved. B) Immunofluorescence of longitudinally imaged lymphatic collecting vessel single plan of WT, Pcsk9$^{-/-}$ and Ldlr$^{-/-}$; hApoB$^{+/+}$ animals. Images were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63x/1.4 oil dic objective. C) Popliteal lymphatic collecting vessels were isolated and digested. LDLR protein was assessed by Western Blot in WT, Pcsk9$^{-/-}$ and Ldlr$^{-/-}$; hApoB$^{+/+}$ mice. LDLR immunoreactivity was observed at 160 kDa. The table on the right confirms equal protein loading.

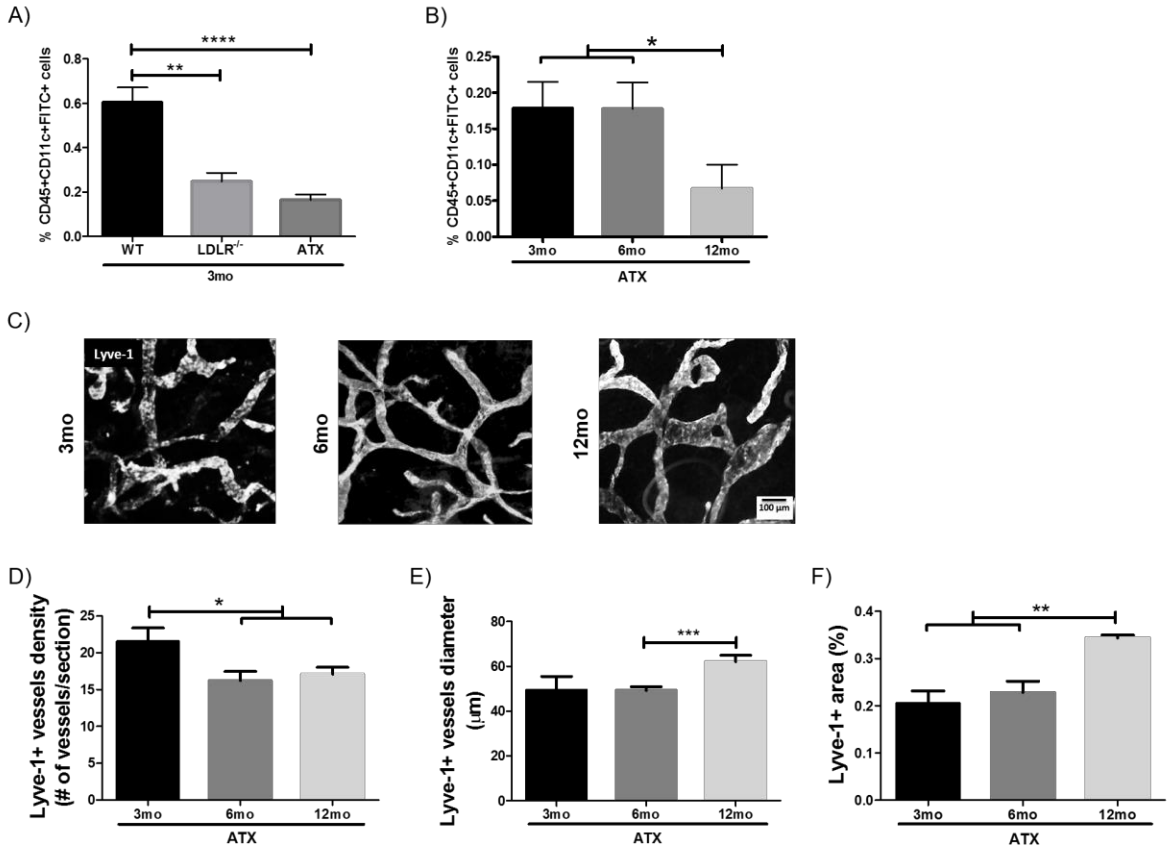


Figure 4. Lymphatic function in the onset and progression of atherosclerosis in ATX mice. Dendritic cell migration was assessed 18h after FITC painting by quantifying %CD45^{+}CD11c^{+}FITC^{+} cells from digested lymph nodes of A) 3-month-old WT, Ldlr$^{-/-}$ and ATX mice, and B) 3-, 6- and 12-month-old ATX mice. Experiments were performed with 5-14 replicates per experimental group. C) LYVE-1 immunostaining was examined in ear whole mounts from Ldlr$^{-/-}$; hApoB$^{+/+}$ mice at ages of 3-, 6- and 12-month-old in ATX mice. Quantification of the D) number E) diameter and F) surface area of Lyve-1^{+} vessels in the ear dermis. Experiments were performed with 4-9 replicates per experimental group (mean of 5 measurements/slide ± SEM). Pictures were taken using Fluoview FV10i. All image handling was performed using ImageJ software. *p<0.05, **p<0.01 and ***p<0.001.

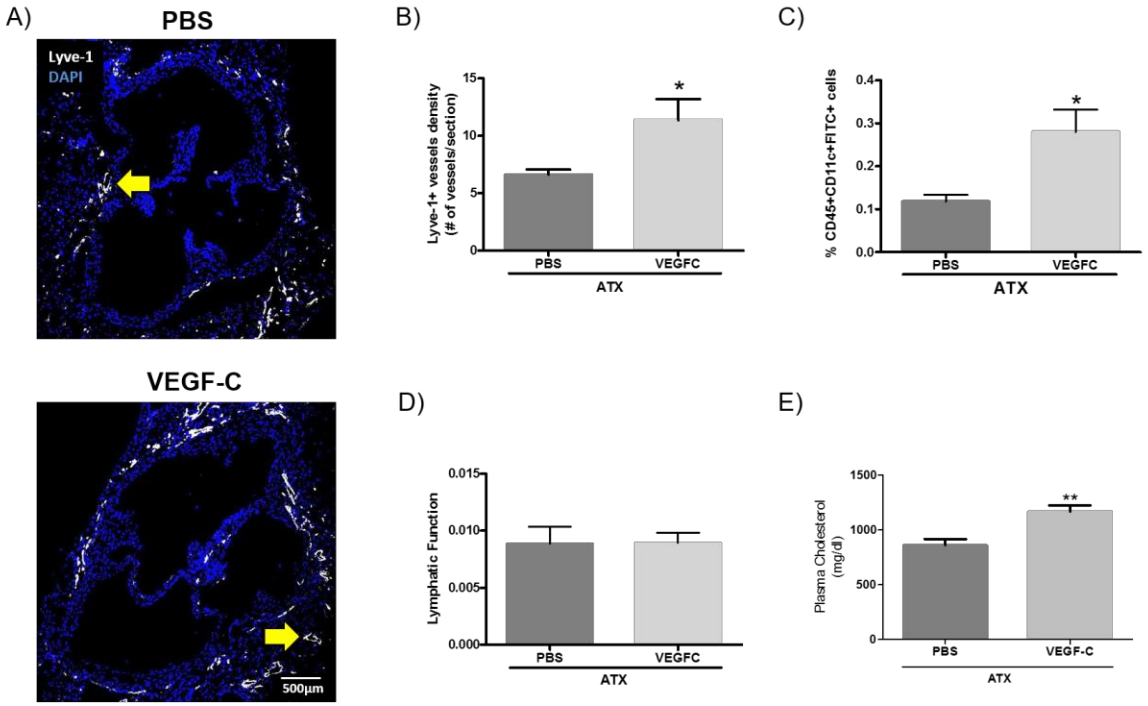
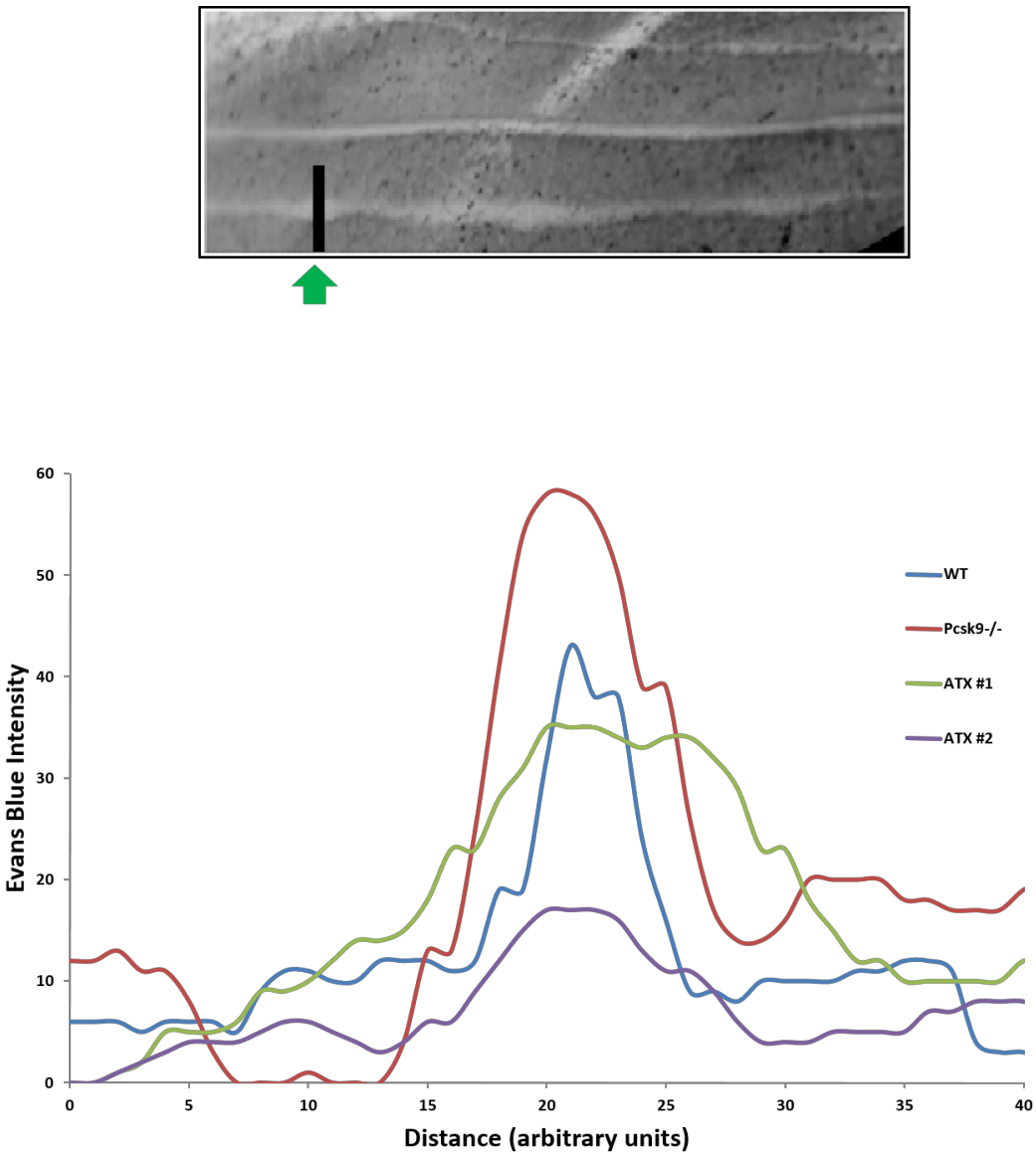
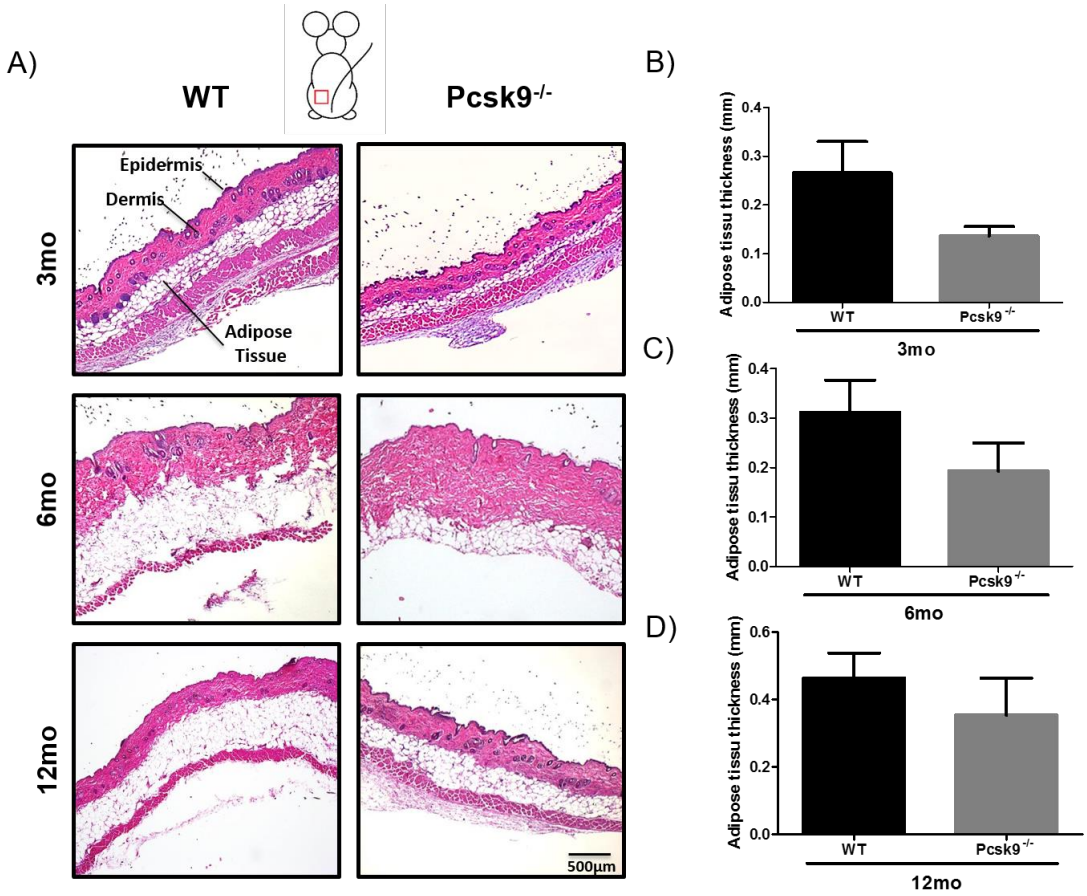


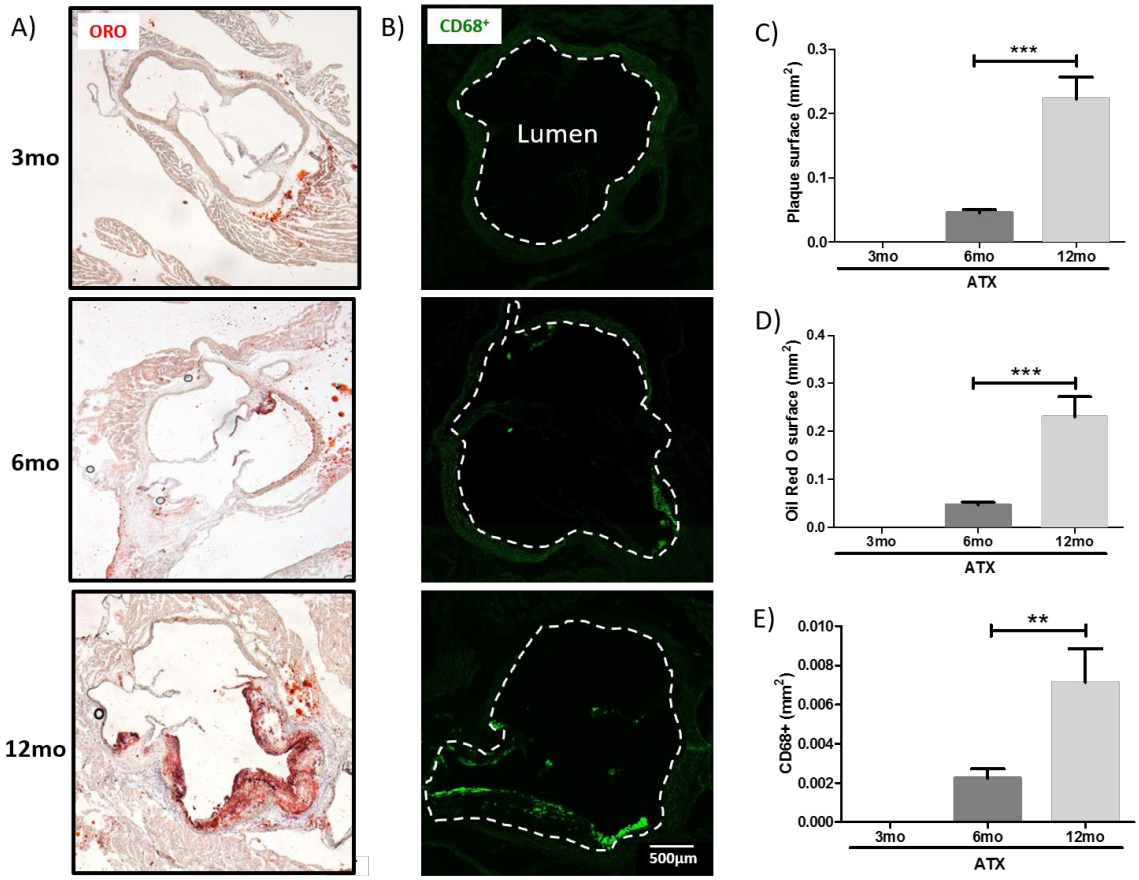
Figure 5. VEGF-C 152s treatment rescues lymphatic cellular transport in pre-atherosclerotic ATX mice. A) Immunofluorescence of initial lymphatic vessels (Lyve-1$^+$) and DAPI of 3 month-old PBS-treated (control) and VEGF-C 152s-treated ATX mice. Only Lyve-1$^+$ vessels of any circular shape were included, as indicated by the yellow arrows. B) Quantification of the number of Lyve-1$^+$ vessels/aortic section. Experiments were performed using 5-7 replicates per experimental group (mean of 2-3 measurements/slide ± SEM). Pictures were taken using Fluoview FV10i. All image handling was performed using ImageJ software. C) Percent of CD45$^+$CD11c$^+$FITC$^+$ cells in skin-draining lymph nodes of 3 month-old PBS-treated (control) and VEGF-C 152s-treated ATX mice. D) Lymphatic molecular transport was assessed by Cy5-labelled Dextran (70 kDa) injection in the ear dermis of PBS- and VEGF-C 152s-treated ATX mice. E) Total cholesterol was measured in plasma of ATX mice treated with VEGF-C 152s or PBS-control. Experiments were performed using 4-5 replicates per experimental group (mean ± SEM) * $p<0.05$.



Supplementary figure 1. *In vivo* semi-quantitative visualization of Evans Blue dye transported through a popliteal lymphatic collecting vessel. Lymphatic function was assessed following Evans Blue dye intradermal injection in mice footpads. After 30 minutes, the dye intensity distribution perpendicular to the popliteal vessels was assessed using ImageJ (as illustrated by the green arrow, upper panel). The histogram (lower panel) illustrates the different intensities of the WT (blue), Pcsk9$^{-/-}$ (red) and the two different ATX collecting vessel phenotypes observed in figure 1A (i.e. leaky (green) and dye-free (purple) collecting vessel segments. Experiments were performed using 2-3 replicates per experimental group.



Supplementary figure 2. Adipose tissue layer from back skin in Pcsk9$^{-/-}$ and WT mice. A) Hematoxylin and eosin staining was performed on 8 µm-thick paraffin skin sections of 3-, 6- and 12-month-old WT and Pcsk9$^{-/-}$. Quantification of adipose tissue thickness in back skin is illustrated B) at 3-, C) 6- and D) 12-month-old mice. Experiments were performed with 3-4 replicates per experimental group (mean ± SEM). Picture were taken with an Olympus B45 microscope and analyzed using ImagePro Plus 7.0 software. All image handling was performed using ImageJ software.



Supplementary figure 3. Plaque development during atherosclerosis progression in ATX mice. Hearts were harvested and immersed in OCT compound. A) Neutral lipid- (Oil Red O) and B) macrophage ($CD68^+$)-positive areas were quantified in 8 µm-think aortic sinus sections of 3-, 6- and 12-month-old ATX mice. C) Plaque surface (mm^2), D) Oil Red O and E) $CD68^+$ area were quantified using ImageJ software. Experiments were performed with 4-9 replicates per experimental group (mean of 2-3 measurements/slide ± SEM). **p<0.01 and ***p<0.001.

3. DISCUSSION

This Mémoire describes important new findings in an emerging field bridging lymphatic function, lipoproteins and atherosclerosis. Our results suggest that lymphatic dysfunction is present before the onset of atherosclerosis, and that this dysfunction is primarily associated with a defect in the collecting lymphatic vessels, thereby limiting lymph transport from peripheral tissues to the blood. Our subsequent work shows that this lack of lymph propulsion could be due to the absence of the LDLR, and that lymphatic transport can be restored by systemic injections of a VEGFR-3-selective agonist, VEGF-C 152s. Our work identifies new potential therapeutic targets for the prevention and treatment of atherosclerosis. This opens doors to the possibility of preventive care, in which patients could be screened for lymphatic dysfunction and obtain a treatment that could hopefully abrogate atherosclerosis development.

Beginning with the therapeutic potential of the PCSK9 ligand, which at the moment is a very prominent subject of interest in the lipoprotein field, we showed that lack of PCSK9 offers increased protection against atherosclerosis. As our results show, the increased presence of LDLR might affect lymphatic function, the nature of which remains to be identified. To our knowledge, we are the first to clearly show by immunofluorescence and western blot that LDLR is present at the surface of LECs, bringing forth new regulatory possibilities for these cells and ultimately, of the lymphatic network as a whole.

Secondly, we demonstrated that early treatment with VEGF-C 152s is able to rescue lymphatic function in pre-atherosclerotic $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice. We need to further study its long-term effects on plaque regression and better characterize its protective outcome. So far, supported by data from the literature, we believe that VEGF-C 152s acts on the capacity of collecting lymphatic vessels to contract, rather than by increasing lymphangiogenesis, as its primary role may suggest.

The origin of the lymphatic dysfunction is now better understood in both pre-atherosclerotic, as well as atherosclerotic mice, as we eliminated the possibility that the lymphatic capillaries are a cause of lymphatic impairment. Due to their normal morphology, as well as function, clearly demonstrated in our mouse models, our data strongly suggests that

a defect predominantly occurs at an upstream level, namely the collecting vessels. Further experiments we are currently working on will better delineate this impairment. In the following sections I will go into more detail explaining our rationale of the steps we have undertaken.

3.1 Pathophysiology of the lymphatic collecting vessels

Dysfunction in lymphatic transport is central to numerous pathologies that affect millions of people worldwide[96]. It now comes as no surprise that this impairment could also be seen in atherosclerosis. In this project, in order to pinpoint the exact portion (i.e. lymphatic capillaries or lymphatic collecting vessels) of the lymphatic vessels that may cause the impaired lymph through the lymphatics, we first started looking at the lymphatic capillaries, which are easy to study as they are located at the extremity of most tissues. When we looked at their morphology (their branch size, diameter, as well as the total surface area they cover) in the ear dermis of the mouse, we were unable to see any differences between $Pcsk9^{-/-}$ and $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice, at their respective age. We did however see differences begin to appear in $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice, as they get older. As it has already been shown that lymphatic capillary integrity diminishes with age[97], we also performed functional tests using a molecular tracer (dextran-Cy5) that is large enough to only be taken up by these lymphatic capillaries (± 240 μm) , but not able to enter the bloodstream. Once again, our results indicated that, at least before the onset of atherosclerosis, the lymphatic capillaries appear to function normally.

Intrinsic and extrinsic factors can limit lymphatic contraction. Anatomical factors include, but are not limited to, the over-accumulation of skin adipose tissue measured in $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice. As previously described, lymphatic collectors are modulated by extrinsic factors such as the SMCs that surrounds them. An increase in fat around the vessels, which suggests a decrease in the muscle surrounding the vessels, may diminish their contractile ability. Furthermore, enlarged adipocytes recruit macrophages and promote inflammation[98], and collecting lymphatic vessel permeability facilitates adipose tissue inflammation and distribution of antigen to lymph nodes[99]. However, we see a reduction in lymphatic drainage probably due to the production of various mediators associated with inflammation such as the production of NO[100,101]. As seen before, under normal physiological conditions, eNOS is the

primary source of NO produced in response to increased shear flow. However, during an inflammatory response, NO can also be produced by immune cells surrounding collecting lymphatic vessels and in adjacent spaces of the interstitium, via iNOS, independent of the endothelium. The resulting excess of NO results in inhibition of the pumping capacity of the lymphatics[102]. Supporting this, Scallan *et al*[73] showed that NO production, whether basal or stimulated, depresses murine collecting lymphatic contractile activity. We therefore expect to see a disturbance in intracellular Ca^{2+} and an overproduction of NO in our atherosclerotic mouse model.

Age is a major driving force for change in the body, and so the effect of age on lymphatic structure and function should also be taken into consideration. Decreased contractility and pumping efficiency, as well as a decrease in extracellular matrix and contractile proteins in aged lymphatic collectors, are believed to be physiological changes that occur with age[103]. Furthermore, impaired pathogen clearance, and impaired permeability, leading to leakiness in aged collectors, were also seen[103].

When it comes to the role of lymphatics in mRCT, and ultimately atherosclerosis, our results indicate that further understanding of the physiological characteristics of lymphatic vessel pumping and general dynamics is required. We are currently studying not only the contraction capacity of the lymphatics, but also their permeability, valves and endothelial junction integrity.

3.2 Potential treatment using a selective agonist of VEGFR-3

This part of the project is inspired by promising preliminary results showing treatment with VEGF-C 152s, a mutated form of VEGF-C that specifically activates VEGFR-3, restores lymphatic molecular transport in the ear dermis of both hypercholesterolemic $apoE^{-/-}$ and $Ldlr^{-/-}$ mice. Based on these results, we went on to investigate if VEGF-C 152s can rescue lymphatic transport in 3-month old $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice: at this age these mice show no atherosclerotic lesions. We initially examined the molecular transport. We did not see any differences following treatment, which we attribute to the fact that at this age, the lymphatic capillaries, which are responsible for absorbing the molecular tracer, are seemingly normal. However, we were able to see an effect of VEGF-C 152s in 3-month old $Ldlr^{-/-}$; $hApoB100^{+/+}$

mice when cellular transport was assessed. Following treatment with VEGF-C 152s, lymphatic function was restored. We excluded the fact that VEGF-C could have decreased total plasma cholesterol (TPC) and subsequently improved lymphatic function, as TPC was significantly higher in VEGF-C-treated mice versus PBS-treated $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice. This led us to conclude that although VEGF-C promotes lymphangiogenesis, it may actually stimulate lymphatic pumping as well. Supporting data from the literature suggest that VEGF-C increases lymphatic contraction frequency, dilation, and pump flow through its action on VEGFR-3. In fact, it is speculated that the lymphatic contractile response to VEGFR-3 activation involves multiple signalling pathways, such as the release of intracellular Ca^{2+} within LECs and activation of eNOS, to regulate phasic and tonic contractility[104]. To date, VEGF-C/VEGFR-3 binding on LECs has been shown to activate PI3K/Akt and increase the phosphorylation of P70S6K, PLCγ1, Erk1/2 and eNOS.

Inflammation is a critical part of the atherosclerotic process, and studies of VEGF-C in inflammatory bowel disease (IBD), a chronic inflammatory disease, could prove to be useful in better understanding atherosclerosis. Crohn's disease, for example, is associated with an aberrant mucosal immune response and furthermore, D'Alessio *et al*. demonstrated that adenoviral induction of VEGF-C expression provides marked protection against the development of acute and chronic colitis in two different animal models. These authors believe that VEGF-C offers protection mediated by 'resolving macrophages' in a STAT6-dependent manner[105]. STAT6 promotes interleukin-4 (IL4) mediated biological responses, which decreases the production of M1 macrophages (pro-inflammatory), thus allowing the alternative activation of repair macrophages, M2 (anti-inflammatory)[105]. This all leads to a decrease in pathological inflammation, suggesting that treatment with VEGF-C may serve to delay the development and/or progression of atherosclerosis. We now aim to further investigate the athero-protective role of VEGF-C in the $Ldlr^{-/-}$; $hApoB100^{+/+}$ mouse model of atherosclerosis.

3.3 LDLR modulation and PCSK9 as a potential therapeutic target

Our results lead us to believe that hypercholesterolemia and/or aging are not the only players responsible for the impaired lymphatic transport impairment in atherosclerotic mice. As our findings point out, despite the absence of atherosclerotic lesions prior to the onset of atherosclerosis, 3-month old $Ldlr^{-/-}$; $hApoB100^{+/+}$ mice display a clear defect in cellular lymphatic transport. On the contrary, $Pcsk9^{-/-}$ mice display no defects in dendritic cell transport through the lymphatics at each age examined and actually displayed enhanced cell transport through the lymphatics at 6 months. Gain-of-function mutations in the gene encoding PCSK9 are linked to familial hypercholesterolemia[106], analogous to LDLR and ApoB. Furthermore, studies indicate that PCSK9 might accelerate atherosclerosis by promoting inflammation, endothelial dysfunction, and hypertension by mechanisms that are independent of the LDLR[107]. As previously mentioned, PCSK9 enhances LDLR degradation, resulting in LDL accumulation in plasma. In fact, the atherosclerosis-protected $Pcsk9^{-/-}$ mice are characterized by a 3-fold increased hepatic LDLR levels and 80% decreased plasmatic LDL[108]. Furthermore, Le May *et al*. observed that PCSK9 expression impacts postprandial triglyceridemia[109], another important cardiovascular risk factor. As important as the presence of LDLR at the surface of hepatocytes is, we found out that it is also on the surface of lymphatic endothelial cells from collecting vessels. This new data suggests that modulation of LDLR could have a direct effect on lymphatic vessel function. Also, supporting data suggests that removal of cholesterol by lymphatic vessels is dependent on the uptake and transcytosis of HDL by other receptors such as SR-B1 expressed on lymphatic endothelium[110]. However, as Temel *et al*. showed that intestinal SR-B1 is not involved in transintestinal cholesterol efflux (TICE), we believe that these results need further confirmation[111]. Nonetheless, as increased presence of LDLR was observed in the intestines at the surface of the lacteals[109], their presence may play a role in lymphatic transport *per se*. Therefore, we thoroughly believe that LDLR plays a functional role at least in the early stage of lymphatic dysfunction in atherosclerosis, and that PCSK9 inhibition may exert an additional pleiotropic effect on atheroprotection. Many pharmaceutical companies now target PCSK9, but it is also believed that its presence in the body is most certainly not coincidental. Although its inhibition offers

important control against hypercholesterolemia, which we will continue to investigate, especially through the link of the LDLR in conjunction with the lymphatic system, it has been shown that upon hepatic damage, patients lacking PCSK9 could be at risk of hypercholesterolemia[112]. Nonetheless, a great deal of emphasis is currently focused on blocking PCSK9 with the help of monoclonal antibodies[113], and there is even an experimental PCSK9 vaccine that induces the body to produce its own anti-PCSK9 antibodies[114]. Furthermore, PCSK9 has been shown to affect the regulation of epithelial Na^+ channel (ENaC) trafficking and therefore modulate epithelial Na^+ absorption, which is critical for blood pressure control[115]. It will be interesting to see if PCSK9 directly modulates Ca^{2+} channels in LECs. Most interestingly, PCSK9 was shown to be expressed endogenously both at the mRNA and protein level in murine peritoneal macrophages[35], and Fazio *et al*. showed for the first time that this PCSK9 expression directly influences atherosclerotic plaque composition with no changes in serum cholesterol levels, suggesting a direct effect of macrophage PCSK9 in inflammation and plaque development. Therefore, several pros and cons need to be further investigated if we are to assure a risk-free potential treatment against naturally occurring proteins within our bodies.

4. CONCLUSION

Based on our data and work from other groups, there is accumulating evidence that enhancing lymphatic transport could limit atherosclerosis progression and favour plaque regression. We have shown that lymphatic transport is impaired in young, atherosclerosis-prone mice, even before the onset of atherosclerosis. We believe it to be potentially associated with a defect in the lymphatic pumping capacity, and we suggest that enhancing VEGF-C/VEGFR-3 signalling can abolish this specific defect. As LDLR appears to be implicated in lymphatic function, we now aim to further study the possible effects of PCSK9 and LDLR modulation, in modifying lymphatic activity.

As the Montreal Heart Institute has a unique infrastructure in Canada to study atherosclerosis in clinical settings (i.e. the Pharmacogenomics Center and BioBanque), we are eager to pursue translational projects to assess the correlation between lymphatic function and atherosclerosis in patients. We are currently optimizing a non-invasive near infrared (NIR) imaging technique that detects exogenous indocyanine green (ICG) in the lymphatic vessel, following its systemic intradermal injection. Our main goal, with the collaboration of local and international experts, is to identify strategies that may enhance lymphatic function and lead to the prevention and regression of atherosclerosis.

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APPENDIX

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Review

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Lymphatic network in atherosclerosis: the underestimated path

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The lymphatic system is a key component of tissue fluid homeostasis. In contrast to the closed and high-pressure blood vascular system, the lymphatic vascular system transports lymph in an open and low-pressure network. A prerequisite player in the transport of immune cells and cholesterol metabolism, it has been understudied until recently. Whereas defects in lymph circulation are mostly associated with pathologies such as congenital or acquired lymphedema, emerging significant developments are unraveling the role of lymphatic vessels in other pathological settings. In the last decade, discoveries of underlying genes responsible for developmental and postnatal lymphatic growth, combined with state-of-the-art lymphatic function imaging and quantification techniques, have matched the growing interest in understanding the role of the lymphatic system in atherosclerosis. With a historical perspective, this review highlights the current knowledge regarding interaction between the lymphatic vascular tree and atherosclerosis, with an emphasis on the physiological mechanisms of this multifaceted system throughout disease onset and progression.

The blood and lymphatic vascular systems are parallel but interdependent networks. The lymphatic system governs the transport of superfluous interstitial fluids from peripheral tissues to the blood circulation, maintaining fluid balance throughout the body. Defects in lymphatic function have been broadly associated with pathologies such as congenital or acquired lymphedema. Although longstanding observations suggested that the lymphatic vasculature could be central in the development of chronic inflammatory diseases, recent publications specifically point out its potential implication in atherosclerosis. In this review, we highlight the current knowledge unraveling the interaction between the lymphatic network and atherosclerosis, with an emphasis on the physiological mechanisms of this intricate system.

Keywords: atherosclerosis • lymphatic endothelium • lymphatic smooth muscle cells • lymphatic vessels

In the 1620s, two important circuits of the bodies were officially discovered: *De Moto Cordis* and *De lactibus sive lacteis venis* were two independently published manuscripts, portraying the blood and the lymphatic circulation, respectively. Albeit interdependent, the blood and the lymphatic vascular networks are two separate circulatory systems running in juxtaposition, having separate, but often interdependent, functions [1]. Four centuries after their first official identification, advances on blood vascular biology far surpass those of lymphatic biology. Why has there been such a gap in the interest given to both particular systems? Whereas tools to study the venous and arterial circulation have been developed at a greater pace, exploring the lymphatic network had its load of difficulties. Until very recently, very few methods had been optimized to fully characterize lymphatic function in health and disease. The urge for a better understanding of

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this complex system became obvious with the report of the ubiquitous presence of those milky-white vessels in nearly all vascularized tissues. Their potential association with diverse severe pathologies was then clearer. The progress that has been made since the turn of the present century is tremendous: new genetic mouse models [2,3] and imaging tools [4,5] developed for both animals and humans have greatly contributed to unraveling the role of the lymphatic system in different pathophysiologic conditions even outside of the fields of lymphedema, such as in atherosclerosis.

This article highlights recent advances in our understanding of the role of the lymphatic vascular tree in cardiovascular diseases, with a specific attention to atherosclerosis. We place particular emphasis on work depicting the functional mechanisms of this complex system throughout disease onset and progression.

Historical perspective

In Italy, 23 July 1622, a professor of anatomy and surgery was meticulously practicing vivisection on a dog. Gasparo Aselli and a group of colleagues were observing recurrent nerves in the animal. Subsequently, in an attempt to watch the movements of the diaphragm in the same operation, he opened the abdomen. Thinking he was dealing with nerves belonging to the intestines, the Italian described what he saw as '(…) cords, exceedingly thin and beautifully white, scattered the whole mesentery and the intestine, starting from innumerable beginnings.' [6]. Therefore, in the first published color-printed illustrations in a medical or anatomical work, Professor Aselli depicted the 'vessels containing white blood' described by Hippocrates in 400 BC [7] as *lacteae venae*, or 'milky veins' [6]. Three decades later, Thomas Bartholin created the term 'lymphatics,' based on his work performed in parallel to the work of others on the thoracic duct in the same period (Olaus Rudbeck, 1630–1702 ; Jean Pecquet, 1624–1674 ; George Joyliffe, 1621–1658) [8]. While discoveries of the 17th century led to the establishment of the anatomical atlas of the lymphatic system, the discoveries from the two following century portrayed the network as a passive draining network, bringing a first insight on the role of the lymphatic system. William and John Hunter pioneered the discovery of the absorptive capacity of the lymphatics in the 18th century [9], while studies on lymph motion through the lymphatic vessels had started to emerge in the 19th century. Lymph composition *per se* was studied concomitantly, first suggesting that lymph is formed as a filtrate of the blood [10]. It is in the second end of that century that Rudolf Virchow investigated the role of lymph nodes as filtering units [11]. Few decades later, in the early 20th century, Florence Rena Sabin brought up the first insight on lymphatic development. She innovatively proposed lymph sacs originate from endothelial cells that outgrow from the veins during early development [12]. We had to wait for a couple of decades before observing lymphatics through lymphography, a method allowing the clinical observation of lymphatic disorders [13].

General anatomy & functions of the lymphatic vessels

The lymphatic system is now recognized as working in close collaboration with the cardiovascular system. The lymphatic network is an essential component of the immune system, playing major roles in host defense and adaptive immunity, as it is the principal route of transport from tissues for antigen and immune cells [14]. Lymphatic vessels are required for the maintenance of fluid homeostasis within the body [15], absorbing lymph through thin-walled and blind-ended lymphatic capillaries (also called initial lymphatics) from the peripheral tissue. Initial lymphatics are highly permeable and constituted of specialized, discontinuous 'button-like' junctions between endothelial cells [16]. Lymphatic capillaries are characterized by an absence of smooth muscle cells (SMCs) and specific expression of LYVE-1 on the lymphatic endothelial cells (LECs). When lymph continues its path from the initial lymphatics, it converges into larger precollecting and subsequently collecting lymphatic vessels [15,17]. Collecting vessels are responsible of maintaining lymph flow through contraction of units called lymphangions. A lymphangion is defined as a vessel segment delimited by two endothelial leaflet valves. In the absence of strong positive pressure leading the lymph toward the vein, these valves allow unidirectional flow in preventing lymph backflow [16]. A basement membrane, podoplanin expression, continuous 'zipper-like' cell–cell junctions and a SMC layer are also distinguishing collecting vessels from lymphatic capillaries. After reaching the lymph nodes (LNs), the afferent collecting vessels become the efferent collecting vessels and, ultimately, the collecting lymphatic vessels converge into the thoracic duct or the right lymphatic trunk, where lymph is finally reaching the venous circulation via the subclavian vein [13,14]. In the intestines, the lymphatic system is essential as it absorbs lipid through entities called lacteals, transporting lipids from the gut into the blood [18,19].

Following up on Sabin's work [12], it has been described that venous endothelial cells (VECs) differentiation into LEC progenitors from the cardinal vein allows lateral sprouting and lymph sacs formation, the latter being characterized by the expression of the transcription factor Prox1 [20]. Prox1$^+$ cells require a paracrine VEGF-C gradient to spread away from

embryonic veins [21]. Subsequently, the lymph sacs undergo extensive remodeling to form a structured vascular network. Lymphatic development and regulation are dependent upon VEGF-C/D and its receptor VEGFR-3 [22], and Prox1 drives VEGFR-3 expression that enables LEC to respond to VEGFR-3 ligands [23].

To properly determine whether lymphatics are active players in cardiovascular disease progression or regression, we must combine morphological observations and lymph composition with functional studies. Several parameters need to be taken into consideration when assessing proper lymphatic function, whether it is at a cellular or molecular level, both under physiological and pathological conditions.

Lymphatic morphology in atherosclerosis

Lymphatic biology and function in heart disease is gaining exponential attention. First studied in the fields of lymphedema and cancer, the lymphatic vascular tree is now broadly studied in acute and chronic inflammatory diseases. Atherosclerosis is a chronic inflammatory disease affecting large- to medium-sized arteries, and driven by two main constituents: macrophages and cholesterol. Macrophages accumulate in the expanding aortic intima, engulf lipids (becoming so-called foam cells) and produce a wide-ranging spectrum of inflammatory mediators [24–27]. Macrophage reverse cholesterol transport (mRCT) is the mechanism by which cholesterol homeostasis occurs in the artery wall, as cholesterol is mobilized from foam cells and subsequently transferred from the peripheral tissue to the liver and feces [28], or directly to the fecal pathway [29]. Both exit of foam cells and cholesterol from the plaques are believed to be essential targets in the reduction of atherosclerosis burden, and risk of coronary events such as plaque rupture [30].

Surprisingly, despite the well-defined roles of lymphatic vessels in preserving fluid balance throughout the body by returning plasma proteins from interstitial spaces back to the blood circulation, only little attention has been given yet to the role of the lymphatic vasculature in the atherosclerotic process. In the last century, only a few avant-garde scientists pointed out the concept that lymphatics, and particulary lymph flow, could influence atherogenesis [31–33]. In a review manuscript published in 1981, Lemole had concatenated important publications in which intimal thickening was observed following lymphatic blockade. He suggested that enhanced stagnation of the interstitial fluid in the arterial wall could be due to lymphostasis, phenomenon in which factors that might contribute to the development of atherosclerosis, such as intimal edema, are present [33]. He concluded by suggesting that further studies in immunology, pathology, lipid metabolism and nuclear medicine were needed to confirm this hypothesis [33]. Thirty years later, several original fundamental studies and clinical observations have been published in this regard.

Interest to lymphatic biology and function in coronary artery disease is growing exponentially. Morphological analysis of the lymphatic vessels within the arterial wall gave the first insight of the association between the lymphatic system and atherosclerosis. In animal models, lymphatic vessels have been consistently observed in the adventitial and periadventitial regions of the artery wall [34,35]. Xu *et al.* suggested that the arrangement of the lymphatic vessels within the artery wall reflects the importance of this complex network in maintaining the drainage of local inflammatory cells and cytokines from peripheral tissue such as the adventitia [36]. In addition of being studied in dog epicardial coronary arteries, rabbit carotid and thoracic aorta, and rat aortic wall after balloon-induced aortic endothelial injury, the presence of lymphatic vessels have also been observed in hypercholesterolemic mice. In apoE$^{-/-}$ fed on a high-fat western diet, lymphatic capillaries have been found in the adventitial layer, waving in and out of the adjacent adipose tissue of the aorta, and to be quite numerous beneath plaques in the aortic sinus [37].

In a clinical setting, Drozdz *et al.* took interest in the presence of lymphatics in the adventitia of the internal carotid artery in humans and showed that the number of adventitial lymphatics increases with severity of atherosclerosis measured as intimal thickness [38]. Where it becomes particularly interesting, is that they stipulate that arteries that are covered by a dense network of lymphatic vessels seem naturally protected against atherosclerosis when compared with those that lack such a network [39]. In addition, Kholova *et al.* have published that lymphatic vessels can also be found in the intima of advanced lesions [40]. On the other hand, other teams observed no [41] or very little [42] lymphatic vessels in the wall of normal or atherosclerotic human epicardial coronary arteries, despite the accentuated presence of VEGF-C [42].

Controversial morphological characterizations described above certainly deserve critical consideration. For example, what does the presence of lymphatic vessels in the adventitia of the aortic wall really means? Are plaque-associated lymphatic vessels friends or foes in atherosclerosis? Research groups are actively seeking for the answers, especially through altering lymphatic function in animal models of atherosclerosis.

Lymphatic function in atherosclerosis

Three decades after Lemole reported observational studies considering a connection between intimal

edema and lymphatics [33], genetic and surgical interventions in animal models have emerged, in the faith of getting better insights on the role of the lymphatic network in atherosclerosis.

Recently, a functional and quantitative study has reported the prerequisite role of the lymphatic system in the removal of cholesterol from the artery wall, putting front stage the importance of the lymphatic network in mRCT [37]. Using a surgical model of aortic transplant from a hypercholesterolemic apolipoprotein E-deficient ($Apoe^{-/-}$) donor to a hypercholesterolemic $Apoe^{-/-}$ receiver in which ApoE vector was injected to induce cholesterol efflux, it has been shown that the pattern of the newly regrown lymphatic vessels posttransplant is influenced by the blood flow through the transplant [37]. The lymphatic vessels thus formed appeared to be atheroprotective: in conditions where lymphatic vessels had fully grown posttransplant, the cholesterol contained in the transplanted artery was able to exit the atherosclerotic lesion. By contrast, partial inhibition of lymphatic regrowth using VEGFR-3 antibody was reflected by retention of cholesterol in the artery wall of the transplanted aortic segment [37].

Genetic manipulations in mice have also brought new insights on the link between impaired lymphatic vessels and atherogenesis. Primary congenital lymphedema (Milroy disease) is a rare autosomal dominant condition caused by mutations in the *vegfr-3* gene [3]. Primary human lymphedema is characterized by a chronic and disfiguring swelling of the extremities. A mouse model to study the physiological regulation of lymph flow and to assess the therapeutic potential of VEGF-C to stimulate lymphatic revascularization has been put forth by Karkkainen *et al.* [3]. Called 'Chy mice' because of their apparent development of chylous ascites after birth, this mouse model of lymphedema has an inactivating *vegfr-3* mutation in their germ line, causing a selective incomplete development of lymphatic vessels dermally and thus swelling of the limbs [2]. Furthermore, despite the complete loss of initial lymphatics in the limbs and ears resulting in impaired cell trafficking through lymph, the scarce lymphatics present in the body trunk reflected a normal dendritic cell transport [43]. An additional mouse model has been reported to inhibit the formation of the dermal lymphatic vasculature [44]. Mice expressing soluble VEGFR-3 under the keratin-14 (K14) promoter (K14-Vegfr-3-Ig) display a neutralized activity of VEGF-C and VEGF-D in the dermal lymphatic vasculature when expressed in mouse epidermis. They, therefore, also show impairment in transport of solutes and dendritic cells from the skin to the corresponding draining LNs [44]. Vuorio *et al.* took advantage of these characterized mouse models and analyzed the effects of the absence of lymphatics on lipoprotein metabolism and atherosclerosis [45]. Crossing each of these two transgenic mouse models baring lymphatic insufficiency with atherosclerotic mice ($Ldlr^{-/-}/Apob^{100/100}$), the group observed a positive correlation between atheroma formation and the absence of lymphatic vessels. They showed that both sVegfr-3×$Ldlr^{-/-}/Apob^{100/100}$ and Chy×$Ldlr^{-/-}/Apob^{100/100}$ mice have increased cholesterol levels leading to accelerated atherogenesis, suggesting that lymphatic vessels have an important role in maintaining proper lipoprotein metabolism and vascular homeostasis [45].

Inflammation being a critical part of the atherosclerotic process, studies of VEGF-C in inflammatory bowel disease (IBD) might be relevant to atherosclerosis. Crohn's disease and several types of intestinal ulcers are diseases associated with an aberrant mucosal immune response. Recently, D'Alessio *et al.* showed remarkable results, which demonstrated that adenoviral induction of prolymphangiogenic factor VEGF-C provides marked protection against the development of acute and chronic colitis in two different animal models [46]. They explained the protective function of VEGF-C as being meditated by the 'resolving macrophages,' as they call them, in a STAT6-dependent manner. This VEGF-C/VEGFR-3 pathway that seems to regulate macrophage plasticity and activation proves hopeful for the correction of defective lymphatic function especially when it comes to the process of plaque formation in atherosclerosis, particularly in mRCT. Another group recently published that lymphatic impairment worsened the atherosclerosis plaque formation in atherogenic $LDLR^{-/-}/ApoB^{100/100}$ mice crossed with transgenic mice baring lymphatic localized insufficiency, without, however, affecting the RCT rate [45]. In addition to being a key element in promoting the cholesterol efflux from the atherosclerotic lesion, the lymphatic network is thought to play a crucial role in the transport of immune cells involved in the inflammatory response driving plaque progression [47].

Lymph composition in lymphatic function

One of the main role of the lymphatic system is to preserve fluid homeostasis within the body [15] by absorbing lymph from the peripheral tissue and bringing it back to the bloodstream. Lymph is thus rich in lipoproteins such as HDL, immune cells, electrolytes, nutrients and antibodies. Lymph composition influences flow rate, and is believed to modulate lymphatic function *per se*.

Since the early 1970s, it was well known that cardiac lymph originates from the interstitial fluid, and in 1972, Ulall *et al.* determined the flow characteristics and composition of normal heart lymph that

would serve as an essential baseline for future observations [48]. The average lymph chloride and sodium concentrations were higher, whereas the potassium concentration was lower, when compared with blood. Furthermore, all the protein fractions were present in cardiac lymph, but in different concentrations and proportions, as the albumin/globulin ratio was higher in cardiac lymph. The cardiac lymph showed a significantly higher mean lactate level when compared with the coronary sinus blood samples, and the cardiac lymph pH was situated at around 8.0 or higher [48]. By 1983, Sloop *et al.* further characterized the chemical composition and physical appearance of peripheral lymph HDL that was markedly different from that of plasma HDL, especially in cholesterol-fed dogs [49,50]. Lymph HDL had higher cholesterol to protein ratio and markedly increased free cholesterol content when compared with plasma HDL. The phospholipid content of lymph HDL was higher than that of plasma HDL, while the protein content was lower.

Although peripheral lymph lipoproteins have been characterized in animals, there is scarce information about their composition, and close to none about their ultrastructure, in normal humans. There are however, some studies that have started to emerge, analyzing human lymph [51]. Several studies have confirmed that lymph composition is different than that of plasma or serum. Back in 2000, Nanjee *et al.* elucidated the fact that the concentration of small prebeta HDLs in human tissue fluids is determined only in part by their transfer across lymphatic capillary endothelium from plasma. They showed that local production, by remodeling of spheroidal HDLs in tissue fluids, is just as important [52]. Continuing down this path, in 2001, Nanjee *et al.* made observations regarding the composition, as well as the ultrastructure of different subclasses of normal human peripheral lymph lipoproteins [53]. Apolipoprotein B was found almost exclusively in low density lipoproteins, and more importantly, total cholesterol concentration in lymph HDL was 30% greater than could be explained by the transendothelial transfer of HDL from plasma, which provided direct confirmation that HDL acquires cholesterol in the extravascular compartment [53].

We now know that a broad array of cytokines, proteins, growth factors are contained within lymphatic fluid, which play an important role in metabolism, proliferation, as well as an immunoregulatory role [54]. When comparing with serum concentrations, Zaleska *et al.* concluded the existence of a local autonomous regulatory humoral mechanism in tissues, not reflected in serum, after assessing the fact that local cells contribute to lymph concentration by own production.

Propelling the lymph down the road: the physiology of the contraction

Lymph production has a significant impact on lymphatic vessels capacity to actively participate to lymph flow [55]. Lymph transport throughout the lymphatic network is regulated by different mechanisms that are either extrinsic or intrinsic to the lymphatic vessels. The relative importance of intrinsic and extrinsic pumping mechanisms varies from a lymphatic bed to another. The extrinsic mechanisms include lymph generation, arteriolar gradient and surrounding muscle contraction (skeletal or smooth muscle). Active transport of lymph is also possible through contraction of collecting lymphatic vessels, considered as intrinsic lymph transport mechanism. Since changes in pressure and flow are both causes and effects of adaptive processes, it becomes crucial to study the adaptation of the lymphatic network. Therefore, a recent study by Dongaonkar *et al.* attested the changes in mesenteric lymphatic muscle mechanical properties and the intracellular Ca^{2+} in response to sustained mesenteric venous hypertension [56]. They showed that following 3 days of mesenteric venous hypertension, the adaptive response of postnodal mesenteric lymphatic vessels resulted in weaker pumps with decreased cytosolic Ca^{2+} concentration. So when it comes to the lymphatics role in mRCT, it is no surprise that further understanding of the physiological characteristics of lymphatic vessel pumping and general dynamics is required.

Although LECs lining the lumen of the vessel are important modulators of lymphatic contraction, lymphatic SMC (LSMC) are described as the active components, generating both force and rhythmicity responsible to lymph flow. LSMCs wrap lymphangions in a disorderly mesh-like structure [57]. Accordingly, lymphatic valves function (open–close transitions) is passive and responds to differential pressure between pre- and post-valve lymphagions [58]. Conversely, lymphangion contraction is an active process, requiring the generation of force by the smooth muscle cells. Such process mainly depends on myocyte intracellular Ca^{2+} levels. As described in the following section, most mechanisms triggering or modulating vessel phasic or tonic contraction are influencing LSMCs cytoplasmic Ca^{2+} levels. Increase in cytoplasmic Ca^{2+} leads to its complexing with calmodulin, which activates myosin light chain kinase (MLCK). MLCK stimulates generation of force by the myosin/actin interactions and movement. As in blood vessels, Ca^{2+} dependence of the contractile apparatus can also be modulated and has a significant impact on myocyte contraction [59,60]. Although currently understudied, this regulatory pathway is nonetheless quite effective. However, its involvement in the regulation of lymphatic vessels

active pumping might be more relevant in pathological conditions.

Lymph flux upstream will increase intraluminal pressure within a lymphangion, producing distension of the vessel wall. Such stretch will trigger a myogenic response from the LSMCs. The ensuing contractions of the smooth muscle cells will then increase intraluminal pressure and propel the lymph through the following valve and lymphangion [61]. Although the specific components integrating vessel wall stretching into contraction and myogenic contraction remains to be clearly established, membrane potential is depolarized in stretched LSMCs [62].

In addition to transluminal pressure, lymphatic contraction is sensitive to lymph flow. Every contraction stroke is associated with a pulsatile fluid movement, a nonlaminar lymph flow due to the presence of valves. The subsequent shear stress stimulates endothelial cells, which modulate LSMCs contraction [63,64]. However, there are several limitations to interpreting the role of shear stress in this process. For example, the impact of shear stress on endothelial activity is generally studied at supraphysiological levels of shear stress. Although shear stress in lymphatic vessels is estimated around 0.6 dyn/cm^2, endothelial activation reaches a plateau when exposed to shear stress higher than 3 dyn/cm^2. These experiments are generally carried out in cultured cells, where phenotype alteration might result in a significant modification of the effect observed. Moreover, loss of communication with the underlying smooth muscle could also be responsible for changes in sensitivity to lymph flow. However, mathematical models strongly suggest that differential flow is responsible for higher NO concentration near lymphatic valves [65].

Lymphatic contraction is essentially depending on intrinsic LSMCs properties. However, the myocytes undergo several regulatory influences, including humoral and neural. The most important modulator of LSMCs contraction is the endothelium as endothelium strongly regulates lymphatic smooth muscle electrical excitability and contractility. This control occurs mainly through the generation and release of nitric oxide (NO), although other endothelial derived vasoactive molecules including but not limited to arachidonic acid derivatives (e.g., prostacyclin, thromboxane A2) and endothelin-1 have also been reported to modulate lymphatic contraction dynamics. However, several other candidates in endothelial-dependent modulation of lymphatic contraction have not been explored yet. For example, local ATP release by Pannexin channels could activate nearby purinergic receptors. Similarly, opening of endothelial K^+ channels such as $K_{Ca}2.3$ or $K_{Ca}3.1$ channels might lead to 'K^+ clouds', activating smooth muscle Kir channels and promoting its relaxation (or a decrease in phasic contractions) via myocyte hyperpolarization. Interestingly, VEGF-C through its binding to VEGFR-3 has also been reported to modulate lymphatic pumping as positive chronotrope and increased lymphagion dilation [66].

Phasic contractions of lymphangions are also spontaneous as they can occur in the absence of stimulation. Autonomous contraction is triggered by LSMCs action potentials (APs). Interestingly, tight electrical coupling between LSMCs allows synchronization of myocytes electrophysiological state and coordinated contraction within individual lymphangions. APs result from the summation of spontaneous transient depolarisations (STDs) occurring in LSMCs and might lead to myocyte contraction [67]. Myocyte stretching results in an increase in STD and AP frequency and the associated contraction. STDs originates from a spontaneous IP_3 receptor-dependent Ca^{2+} release that activates Ca^{2+}-activated Cl^- channels (ClcCa) [62]. Molecular identity of ClcCa remains to be established but growing body of evidence suggests that vascular ClcCa might be encoded by TMEM16A. As of now there is no information available on the impact of the loss of TMEM16A expression on lymph propelling, but deleterious effects on lymphatic function can be hypothesized. Likewise, pathological conditions associated with dysfunctional lymphatic vessel contraction could involve alteration of TMEM16A functional expression. On the other hand, Bestrophins can encode Ca^{2+}-activated Cl^- channels and might also represent lymphatic ClCa, stressing out the requirement of further investigation. Despite its unresolved identity, repolarizing currents by Clca will increase open probability of L-type Ca^{2+} channels, increasing Ca^{2+} influx and leading to contraction. Similarly to blood vessels, the main voltage-dependent Ca^{2+} channel involved appears to be Cav1.2 but a contribution of T-type Ca^{2+} channels to lymphatic pacemaking capacity have also been reported [68]. Several other ion channels recently found to be important in blood vessels (e.g., two-pores K^+, KCNQ channels) have not been studied in lymphatic vessels. Their potential absence in physiological condition does not warrant unequivocal exclusion of pathological involvement.

The mechanisms responsible for the spontaneous ER Ca^{2+}-store release leading to STDs remain to be established. A Ca^{2+}-induced Ca^{2+} release coupling might be involved, where small Ca^{2+} entry would favor IP_3R opening and Ca^{2+} release [69]. Alternatively, endothelial Ca^{2+} influx pathways in lymphatic vessels are currently unknown other than the voltage-dependent Ca^{2+} channels. Recent exciting work on local Ca^{2+} dynamics in blood vasculature brings enthusing hypotheses. Indeed, local Ca^{2+} entry through TRP channels such as TRPV4 [70] or TRPA1 [71] could lead to local Ca^{2+}

increase, with little effect on global Ca^{2+}. Moreover, Ca^{2+} microdomains appears to be tightly regulated by multiprotein complexes [71,72]. Local Ca^{2+} entry through voltage-dependent Ca^{2+} channels (like Ca^{2+} sparklets) might also be involved like in blood vessels.

Despite the fact that each lymphangion contraction dynamic is independent (they can be autonomous), there is strong evidence that interlymphangions electrical communication allows a synchronization of the vessel contraction and improves stroke efficiency [57]. Intercellular communication is relying on expression of gap junction channels, complexes of connexins, well characterized in lymphatic and blood vessels [73,74]. Interestingly, electron microscopy studies showed the presence of myoendothelial projections (MEPs) through the basal lamina [75]. These MEPs are in fact privileged sites of communication between endothelium and myocytes, where clustering of specialized protein complexes allow specific signaling and microdomains. Direct electrical coupling does not seem to occur [76] but is not necessary to endothelial-smooth muscle communication as diffusible molecules can be involved, as 'K^+ clouds' or even NO production in these juxtaposed structures.

Conclusion & future perspective

Tremendous progresses have been made toward understanding the mechanisms bridging lymphatic biology to chronic inflammatory disease such as atherosclerosis. Development of genetic mouse models, surgical methods and functional tools has contributed to the exponential advances made in the field in the last decades. Notwithstanding of the significant advances, it is clear from the research highlighted in this article that there is still much to learn about the role of the lymphatic network in coronary heart disease. In the near future, new therapies targeting the functional link between lymphatic dysfunction and atherosclerosis are predicted to emerge with continuously promis-

Executive summary

Background

- Tools to study the venous and arterial circulation have been developed at a greater pace, while exploring the lymphatic network had its load of difficulties.
- Tremendous progress in lymphatic biology has been made since the turn of the present century: new genetic mouse models and imaging tools, developed for both animals and humans.
- This article highlights how progress has contributed to unraveling the role of the lymphatic system in different pathophysiologic conditions, such as in atherosclerosis.

Historical perspective

- While practicing vivisection on a dog, Gasparo Aselli and a group of colleagues depicted the 'vessels containing white blood' described by Hippocrates in 400 BC.

General anatomy & functions of the lymphatic vessels

- The lymphatic network is an essential component of the immune system, and is required for the maintenance of fluid homeostasis within the body.
- Lymphatic vessels absorb lymph through blind-ended lymphatic capillaries, which then converges into larger precollecting and subsequently collecting lymphatic vessels, responsible of maintaining lymph flow through contraction of units called lymphangions.

Lymphatic morphology in atherosclerosis

- Lymphatic vessels are present in the aortic wall, mostly in the adventitia.

Lymphatic function in atherosclerosis

- Recently, a functional and quantitative study has reported the prerequisite role of the lymphatic system in the removal of cholesterol from the artery wall, putting front stage the importance of the lymphatic network in mRCT.

Lymph composition in lymphatic function

- Lymph is rich in lipoproteins such as HDL, immune cells, electrolytes, nutrients and antibodies.
- Lymph composition influences flow rate and is believed to modulate lymphatic function *per se*.

Propelling the lymph down the road: the physiology of the contraction

- Transport throughout the lymphatic network is regulated by different mechanisms that are both intrinsic and extrinsic to lymphatic vessels.
- Lymphangion contraction is an active process, requiring the generation of force by smooth muscle cells and modulated by the endothelium.

Conclusion & future perspective

- Tremendous progresses have been made toward understanding the mechanisms bridging lymphatic biology, to chronic inflammatory disease such as atherosclerosis.
- Translational studies need to further evolve, in order to soon be able to use lymphatic dysfunction as a clinical marker essential for cardiovascular diseases prevention.

ing results, bringing forward novel perspectives for the treatment of atherosclerosis.

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